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UNIVERSITY OF ALBERTA

I. SOME OLD AND SOME NEW *LYCOPODIUM* ALKALOIDS
II. THE TENUAZONIC ACID - *MYCENA CITRICOLOR* INTERACTION

BY

ALBERT WARREN ELGERSMA ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
PARTIAL FULFILMENT OF THE REQUREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA
FALL 1990

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THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED I. SOME OLD AND SOME NEW *LYCOPODIUM* ALKALOIDS II. THE TENUAZONIC ACID - *MYCENA CITRICOLOR* INTERACTION SUBMITTED BY A.WARREN ELGERSMA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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Date: October 5 1990

To Susan

ABSTRACT

The pioneering work on *Lycopodium* alkaloids carried out by R.H.F. Manske and L. Marion led to the characterization of a large number of alkaloids. Many of these alkaloids were designated by numbers preceded by the letter L. In some cases an L numbered alkaloid isolate comprised a single compound, while in other cases, an L numbered alkaloid was shown to be a mixture. While the identity of many of these alkaloids has now been established, several remain unidentified. Samples of L15, L16, L17, L18, L22, L24, L25, L26, L28, L29, L31, and L35 obtained from Marion's collection were examined by GC-MS and GC-IR. The identity of these alkaloids and alkaloid mixtures are reported herein, as well as a complete listing of the L numbered alkaloids. The significance of these alkaloids to the taxonomy of the species from which they were isolated is discussed.

The alkaloids of *Lycopodium meridionale* were examined and three were isolated and identified. These include the previously known alkaloid lycocernuine, an isomer of dihydroallocernuine identified for the first time as a natural product, and a previously unidentified alkaloid which we call lycomeridine.

In Costa Rica and other areas of Central and South America, the coffee pathogen, *Mycena citricolor,* causes severe losses in coffee production. It has been known for some time that mycelium of *M. citricolor* fungus can be induced to produce mushrooms in co-culture with a variety of other fungi, including *Alternaria alternata*. In this thesis, the metabolites of *A. alternata* were examined in an attempt to isolate the active substance. The previously reported tetramic acid, tenuazonic acid, which was isolated from *A. alternata*, stimulated mushroom production and inhibited mycelial growth of *Mycena citricolor*. Tenuazonic acid ((+) form, (-) form, (+/-) form) was prepared by synthesis and it was shown that the (-) form showed the greatest activity.

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TABLE OF CONTENTS

Part 1: Some Old and Some New *Lycopodium* Alkaloids

Introduction

A) General Introduction ---------- 1

B) The 'L' Numbered Alkaloids ---------- 11

C) *Lycopodium meridionale* ---------- 13

Results and Discussion

A) The 'L' numbered Alkaloids ---------- 16

B) *Lycopodium meridionale* ---------- 33

Experimental

A) General ---------- 70

B) The 'L' numbered Alkaloids ---------- 72

C) *Lycopodium meridionale* ---------- 85

References ---------- 98

Part 2: The Tenuazonic Acid - *Mycena citricolor* Interaction

Introduction ---------- 103

Results and Discussion ---------- 108

Experimental ---------- 133

References ---------- 155

LIST OF TABLES - PART 1

Table

1. The *Lycopodium* species of Canada ---------- 6

2. Wilce's classification of the *Lycopodiaceae* Family ---------- 7

3. Grouping of *Lycopodium* species based on alkaloid type ---------- 8

4. The unidentified 'L' numbered alkaloids of Marion and Manske ---------- 12

5. *Lycopodium* alkaloids found in the sub-genus *Lepidotis* ---------- 14

6. ^{1}H NMR decoupling data ($CDCl_3$) for 5,15-oxidolycopodane ---------- 24

7. ^{1}H NMR nOe data ($CDCl_3$) for 5,15-oxidolycopodane ---------- 24

8. ^{1}H NMR COSY data ($CDCl_3$) for 5,15-oxidolycopodane ---------- 25

9. Alkaloid skeletal types present in the *Lycopodium* species examined by Marion and Manske ---------- 28

10. The 'L' numbered Alkaloids ---------- 29

11. ^{1}H NMR spectral data ($CDCl_3$) for lycocernuine ---------- 34

12. ^{1}H NMR spectral data ($CDCl_3$) for cernuine ---------- 35

13. ^{13}C NMR spectral data ($CDCl_3$) for cernuine and lycocernuine ---------- 35

14. Spectral comparisons between LM-1 and lycocernuine ---------- 36

15. ^{1}H NMR solvent induced shifts for lycocernuine and LM-1 ---------- 37

16. Mass spectral data for LM-1 and lycocernuine (HREIMS) ---------- 39

17. ^{1}H NMR nOe data (benzene d6) for LM-1 ---------- 45

18. ^{1}H decoupling data (benzene d6) for LM-1 ---------- 45

19. ^{1}H NMR nOe data ($CDCl_3$) for LM-3 ---------- 55

20. ^{1}H decoupling data for LM-3 ---------- 56

21. ^{13}C NMR spectra data for LM-3 and dihydroallocernuine at 25°C and 55°C ---------- 59

22. Comparison of some ^{13}C NMR signals between cernuine and lycocernuine ---------- 67

23. ^{13}C NMR assignments of some alkaloids with the cernuane skeleton ---------- 69

24. Compounds in the GC/MS and GC/IR library ---------- 84

LIST OF TABLES - PART 2

Table

1. Spectral data for tenuazonic acid and the mushroom stimulating metabolite --- 115
2. Extract yields from culture fluids of *Alternaria alternata* ---------- 137
3. Biological screening of extracts from *A. alternata* ---------- 137
4. Time study of *M. citricolor* mushroom stimulation by *A. alternata* ---------- 138
5. Biological screening of extracts from *A. alternata* shake culture ---------- 139
6. Screening of the separated methanol fractions for biological activity ---------- 140
7. Biological screening of the products from test sample 5 (Table 6) ---------- 140
8. Concentration effects of (-)-tenuazonic acid on *Mycena citricolor* after 30 days ---------- 145
9. Concentration effects of (+/-)-tenuazonic acid on *Mycena citricolor* after 7 weeks using the multi-well method ---------- 146
10. Concentration study of (+/-)-tenuazonic acid on *Mycena citricolor* after 6 weeks using the half plate method ---------- 147
11. Concentration studies of natural (-)- and (+/-)-tenuazonic acid on the growth and stimulation of *Mycena citricolor* ---------- 148
12. Inhibition of *M. citricolor* by synthetic (-)-tenuazonic acid after 7 and 9 days ---------- 149
13. Mushroom stimulation by synthetic (-)- and (+/-)-tenuazonic acid using the multi-well method ---------- 150
14. Effects of synthetic (-)-, (+)- and (+/-)-tenuazonic acid on *Mycena citricolor* -- 151
15. Mushroom stimulation of other strains of *Mycena citricolor*. ---------- 152
16. *Mycena citricolor* versus *Penicillium oxalicum* and *Talaromyces flavus* ------- 153
17. *Mycena citricolor* versus culture fluid extracts of *Penicillium oxalicum* and *Talaromyces flavus* ---------- 154

LIST OF FIGURES- PART 1

Figure

1. Family *Lycopodiaceae* 5
2. Alkaloid skeletal types 9
3. *Lycopodium* alkaoid structures from the sub-genus *Lepidotis* 14
4. Mass fragmentation of the 'bridge' of L28-2 (5,15-oxidolycopodane) 22
5. ^{1}H NMR assignments for dihydrolycopodine and 5,15-oxidolycopodane 23
6. ^{13}C NMR assignments for dihydrolycopodine and 5,15-oxidolycopodane 23
7. Structures of the 'L' numbered alkaloids 31
8. The proposed structure of LM-1 38
9. Numbering scheme for LM-1 42
10. Some ^{1}H NMR nOe's observed for LM-1 43
11. ^{1}H NMR assignment for LM-1 44
12. ^{1}H NMR COSY spin systems for LM-1 46
13. ^{1}H NMR COSY spectrum (benzene d_6) of LM-1 47
14. Partial ^{1}H NMR assignment for LM-1-A 48
15. Partial ^{1}H NMR assignment for LM-1-A-H 49
16. Partial ^{1}H NMR spectral assignment for O-acetylcarolinianine 49
17. Acid catylized formation of LM-1 50
18. Oxidation of LM-1 with mercuric acetate 51
19. ^{13}C NMR spectra ($CDCl_3$) of LM-3 at 25°C and 55°C 54
20. Partial ^{1}H NMR assignment for LM-3 55
21. ^{1}H NMR ($CDCl_3$) specta of LM-3 at 25°C and 55°C 60
22. ^{1}H NMR specta ($CDCl_3$) of dihyroallocernuine at 25°C and 55°C 61
23. ^{13}C NMR specta ($CDCl_3$) of dihyroallocernuine at 25°C and 55°C 62
24. ^{13}C NMR specta ($CDCl_3$) of LM-3 and dihyroallocernuine at 55°C 63

25. ^{1}H NMR specta of dihyroallocernuine with addition of
trifloroacetic acid at 25°C and dihyroallocernuine at 55°C ---------------------- 64

26. ^{1}H NMR COSY spin systems for dihydroallocernuine ($CDCl_3$+TFA)----------- 65

27. Dihydroallocernuine ^{1}H NMR assignments-- 65

28 Configurations for the proposed structures of LM-3 and dihydroallocernuine -- 66

LIST OF FIGURES - PART 2

Figure

1. Some toxic metabolites found in the *Alternaria* species-------------------------- 107

2. The main active substance responsible for fruiting in *S. commune* ------------- 108

3. ^{1}H NMR assignment of tenuazonic acid in pyridine-d_5 ------------------------- 116

4. Tautomers of tenuazonic acid and their ^{1}H NMR signals in $CDCl_3$------------- 116

5. ^{1}H NMR assignment of allo-tenuazonic acid in pyridine-d_5 --------------------- 117

6. Isomers of tenuazonic acid -- 120

7. A comparison of the effects of
(-)- and (+/-)-tenuazonic acid on *M. citricolor* ------------------------------------ 124

8. A comparison of the effects of
synthetic (+)-, (+/-)-, and (-)-tenuazonic acid on *M. citricolor* ----------------- 127

9. Bioassay methods --- 135

LIST OF SCHEMES - PART 1

Scheme

1. Mass fragmentation pathways for lycocernuine ---------------------------------- 40

2. Mass fragnmentation pathways for LM-1 -- 41

3. Mass fragmentation of LM-3-- 52

4. The synthesis of dihydroallocernuine-- 58

LIST OF SCHEMES - PART 2

Scheme

1. The isolation procedure for the mushroom stimulating compound -------------113

2. Synthesis of (+/-)-tenuazonic acid--118

3. Improved method for the synthesis of tenuazonic acid --------------------------119

LIST OF PLATES - PART 1

Plate

1. *Lycopodium annotinum* found growing near Swan Hills, Alberta, 1988 ---------- 2
2. *Lycopodium obscurum* ---------- 2
3. *Lycopodium clavatum* ---------- 3
4. *Lycopodium complanatum* ---------- 3

LIST OF PLATES - PART 2

Plate

1. Co-culture of *Mycena citricolor* with *Alternaria alternata* ---------- 106
2. An example of the multi-well method ---------- 112
3. *Mycena citricolor* with natural (-)-tenuazonic acid [4.90 x 10^{-3}M] ---------- 122
4. *Mycena citricolor* with natural (-)-tenuazonic acid [2.41 x 10^{-3}M] ---------- 122
5. *Mycena citricolor* with natural (-)-tenuazonic acid [1.21 x 10^{-3}M] ---------- 123
6. *Mycena citricolor* with natural (-)-tenuazonic acid (Closeup of Plate 4) ---------- 123
7. *Mycena citricolor* with synthetic (-)-tenuazonic acid [1.69 x 10^{-3}M] ---------- 126
8. *Mycena citricolor* with synthetic (-)-tenuazonic acid [0.85 x 10^{-3}M] ---------- 126
9. *Mycena citricolor* on co-culture with *Penicillium oxalicum* ---------- 131
10. *Mycena citricolor* on co-culture with *Talaromyces flavus* ---------- 131

INTRODUCTION - Some Old and Some New *Lycopodium* Alkaloids

A) General Introduction

The genus *Lycopodium* or "club moss" is a remnant of a plant division which was very abundant more than three hundred million years ago. In the coal beds formed by ancient species of these plants, trunks 40 meters in length have been found. Today, the *Lycopodium* species are tiny in comparison to their ancestors, growing to a maximum of only a few feet in height, with many species reaching a maximum height of only eight to ten inches [1].

The *Lycopodium* club mosses are typically low growing and evergreen. The term "club moss" becomes apparent from their appearance, which is coarsely moss-like with club-shaped cones (see Plate 1). There are over 250 species of *Lycopodium*. Most are terrestrial, but some are epiphytic, that is, they grow on other plants but are not attached parasitically. All *Lycopodium* species of the *Phlegmaria* subsection (see Table 2) are epiphytic. The roots of all species are wiry, and may be attached to aerial stems or arise from specialized subterranean stems [2]. *Lycopodium selago* and *L. obscurum*, for example, have roots directly attached to aerial stems and have no long underground stems (see Plate 2). *Lycopodium clavatum* and *L. complanatum*, on the other hand, have roots attached to long subterranean stems which can grow up to 30 feet in length and erect aerial stems are found at intervals along the underground stems (see Plates 3 and 4).

The reproductive cycle of club mosses is varied. Reproduction can take place by means of bulbils or spores. Bulbils are small lateral leafy stem-structures which occur in place of a leaf and which upon becoming detached, may develop into new plants [3].

Reproduction by spores can be a long process. The genus is homo-sporous, that is, all the spores are the same, there are no male and female spores. The spores are found in the axils of leaf-like sporophylls similar to the vegetative leaves (eg., *L. selago)* or in the club shaped cones found on the tip of the stem (eg., *L. annotinum)*. The place of

Plate 1: *Lycopodium annotinum* found growing near Swan Hills Alberta, August, 1988.

Plate 2: *Lycopodium obscurum* ("Tree Club-moss")

Plate 3: *Lycopodium clavatum* ("Wolf's claw")

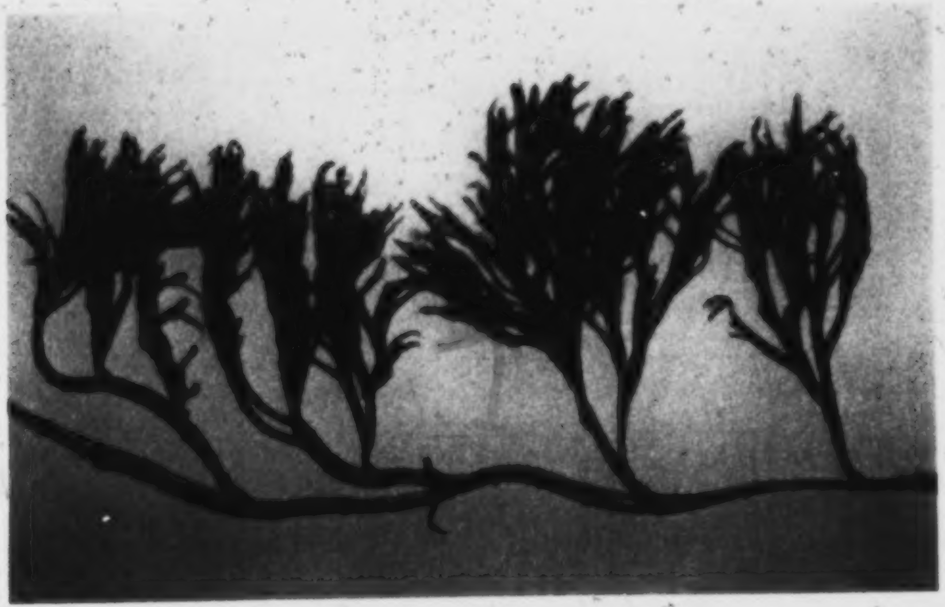


Plate 4: *Lycopodium complanatum* ("Flattened Ground-pine")

germination of the spores as well as the germination time varies with the species. Some spores are short lived and germinate on the surface of the soil (eg., *L. cernuum*), while others can remain dormant for 15 years or more, and only germinate underground in the dark (eg., *L. clavatum*) [4].

After germination, gametophytic tissue "the prothallus" develops. They can be green and photosynthetic if they live on the ground surface, or can be colorless if they live underground. All species, whatever their habitat, depend on mycorrhizal fungi for their development. The prothallus gives rise to sexual organs called antheridia and archegonia. When fertilization of an egg in an archegonium by a sperm from an antheridium is complete, a process which may take up to 15 years, a sporophyte forms which develops roots and leaves and eventually develops into a mature plant [5].

The germination time of the spores appears to be the main factor in determining the type of prothallus that forms. Quickly germinating spores give rise to green prothalli growing on the ground surface. When the spores take a long time to germinate, they become covered with vegetative debris before germinating, and colorless subterranean prothalli develop. The kind of prothallus observed for a species was a characteristic suggested for use as a taxonomic classification [6] until the discovery that "the appearance of the prothallus is not a specific character, but one which could be modified by different environmental conditions" [7]. Spores that are slow to germinate have rough surfaces compared to quickly germinating spores. It has been suggested that these spores are more hydrophobic, or are harder to open than the smoother, quicker germinating spores. Subjecting spores that are slow to germinate to treatment with sulfuric acid or to grinding with sand in dilute detergent apparently weakens or damages the casing around the spore. Germination is then rapid. The prothalli produced in this way are almost identical to those species with faster germinating spores [8]. This is important taxonomically, as well as for those interested in the cultivation of *Lycopodium* species.

Club mosses belong to the family Lycopodiaceae (Figure 1). This group contains two genera of living plants, *Lycopodium* and *Phylloglossum*. There is also one fossil genus, *Lycopodites*. *Phylloglossum* is monotopic; the single species *P. drummondii* is restricted to New Zealand, Tasmania and temperate regions of Australia. *Lycopodium* on the other hand is subdivided into three or four sub-genera, and consists of over 250 species. The majority are tropical, but a large number occur in arctic and alpine regions [9]. In Canada, 13 species of *Lycopodium* have been identified (Table 1) [10].

Figure 1: Family *Lycopodiaceae*.

- ***Lycopodiaceae***
  - ***Lycopodites***
    - no living species
  - ***Phylloglossum***
    - *P. drummondii*
  - ***Lycopodium***
    - *Urostachys* ....*Huperzia*
    - *Lepidotis*...........*Lycopodialla*
    - *Lycopodium*.......*Lycopodium*
    - ....*Diphasiastrum*

Much work has been done in the past to classify and group the known plant species that fall under the description of *Lycopodium* [6,10-18]. However, the taxonomy of the *Lycopodium* species has been under debate since the first classification of the species. "There has been little agreement among botanists concerning the placement of these plants or assignment of species to groups [19]." Some of the recent work by Wilce (1972)[14] and Ollgaard (1975) [13] divide the genus *Lycopodium* into three major groups (*Lycopodium*, *Urostachysis* and *Lepidotis*) (Table 2). More recent work by Cody and Britton (1989) [10] base their classification on four major groups accepted by European workers (*Lycopodium*, *Diphasiastrum*, *Lycopodiella* and *Huperzia*). The grouping of the species into categories has been done using sporophyte morphology (Ollgaard 1975), gametophyte morphology (Rothmaler 1944 [16], Bruce 1976 [12]), spore morphology (Wilce 1972), and chromosome numbers (Love 1977 [11]).

Table 1: The *Lycopodium* Species of Canada

A. *Lycopodium* sub-species group

- 1 *Lycopodium clavatum* L. var. *clavatum*
  - *L. clavatum* L. var. *integerrimum* Spring
- 1.1 *Lycopodium clavatum* L. var. *monostachyon* Hook & Grev.
  - *L. clavatum* L. var. *megastachyon* Fern. & Bissell
  - *L. clavatum* L. var. *brevispicatum* Peck
- 2 *Lycopodium annotinum* L.
- 3 *Lycopodium dendroideum* Michx.
  - *L. obscurum* L. var. *dendroideum* (Michx.) D.C. Eat.
- 4 *Lycopodium obscurum* L. var. *obscurum*
- 4.1 *Lycopodium obscurum* L. var. *isophyllum* Hickey

B. *Diphasiastrum* group

- 5 *Lycopodium complanatum* L.
  - *Diphasium complanatum* (L.) Rothm.
  - *Diphasiastrum complanatum* (L.) Holub
- 6 *Lycopodium digitatum* A. Braun
  - *L. flabelliforme* (Fern.) Blanch.
  - *L. complanatum* L. var. *flabelliforme* Fern.
  - *L. complanatum* L. var. *dillenianum* Doll
  - *Diphasiastrum digitatum* (A. Braun) Holub
  - *Diphasium flabelliforme* (Fern.) Rothm.
- 7 *Lycopodium tristachyum* Pursh
  - *Diphasiastrum tristachyum* (Pursh) Holub
- 8 *Lycopodium alpinum* L.
  - *Diphasiastrum alpinum* (L.) Holub
- 9 *Lycopodium sichense* Rupr.
  - *L. sabinifolium* Willd. var. *sitchense* (Rupr.) Fern.
  - *Diphasiastrum sitchense* (Rupr.) Holub
  - *Diphasium sitchense* (Rupr.) Love & Love
- 10 *Lycopodium sabinifolium* Willd.
  - *Diphasiastrum sabinifolium* (Willd.) Holub

C. *Lycopodiella* group

- 11 *Lycopodium inundatum* L. var. *inundatum*
  - *Lepidotis inundata* (L.) C. Borner
  - *Lycopodiella inundata* (L.) Holub
- 11.1 *Lycopodium inundatum* L. var. *bigelovii* Tuckerm.

D. *Huperzia* group

- 12 *Lycopodium lucidulum* Michx.
  - *Huperzia selago* (L.) Bernh. ssp. *lucidula* (Michx.) Love & Love
- 13 *Lycopodium selago* L. ssp. *selago*
  - *L. selago* L. var. *appressum* Desv.
  - *Huperzia selago* (L.) Bernh.
- 13.1 *Lycopodium selago* L. ssp. *patens* (Beauv.) Calder & Taylor
- 13.2 *Lycopodium selago* L. ssp. *miyoshianum* (Makino) Calder & Taylor

Table 2: Wilce's classification of the *Lycopodiaceae* family.

Lycopodiaceae			
Lycopodium			***Phyloglossum***
Lycopodium	***Lepidotis***	***Urostachys***	*P. drummondii*
Sect.1: Lycopodium	Sect.1: Cernua	Sect.1: Phlegmaria	
Sect.2: Complanata	Sect.2: Lateralia	Sect.2: Selago	
Sect.3-7: Not formally named	Sect.3: Inundata		
Includes:			
Group Fastigiatum			
Group Scariosum			
Group Volubile			
L. deuterodensum			
L. casuarinoides			

Members of the genus *Lycopodium* are a rich source of diverse alkaloids and because traditional characters do not provide a stable classification for *Lycopodium* species, it has been suggested that the diversity of alkaloid content could be used as a source of taxonomic data [20,21]. Since the known *Lycopodium* alkaloids can be divided into 20 different ring systems, the ring systems could be used as classification markers. A successful demonstration of this application is given by Braekman [21].

Braekman re-examined the alkaloid content of some 40 different species of *Lycopodium*. Species were grouped according to the classification proposed by Wilce (Table 2), and the alkaloid content of each of the species was examined for unique characteristics that would coincide with the taxonomic classification. It was found that the alkaloids could be organized into eight different skeletal types which could be used as classification markers .

Three major groups were formed which could be distinguished based only on the type and content of the alkaloids present among different species. The first group includes species of the the sub-genus *Urostachys* which are notable for containing appreciable amounts of alkaloids of type VIII (Figure 2). Species in this group also contain alkaloids of the types II to VI.

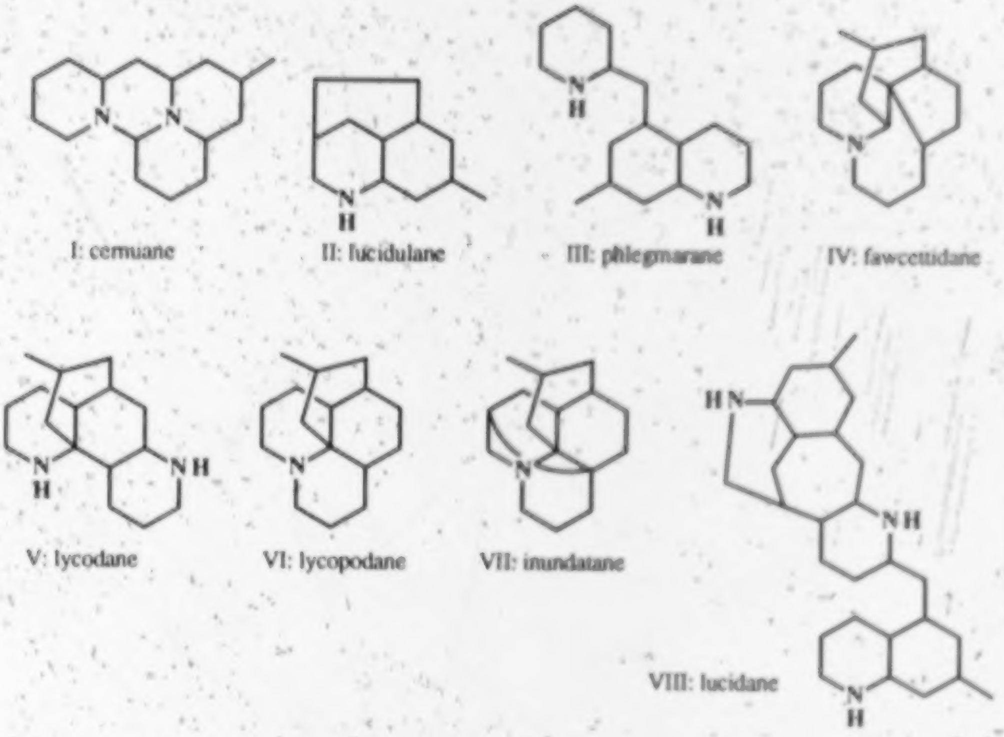
The second group contains species of the sub-genus *Lepidotis* which distinguish themselves by having alkaloids of the type I or VII. Species in this group also have alkaloids of the types III, IV and VI.

The last group contains species of the *Lycopodium* sub-genus, and is characterized by a high content of alkaloids of the type VI. Within this group three sub-groups can be recognized on the basis of the other alkaloid types found in addition to type VI alkaloids. The first sub-group (A) contains species in the sub-section *Volubile* and *L. deuterodensum* and possesses lesser amounts of types III, IV and V alkaloids. The second sub-group (B) contains species in the sub-section *Fastigiatum* and possess small amounts only of type IV alkaloids. The third sub-group (C) contains species in the section *Complanata* and possesses small amounts of type IV and type V alkaloids. This last sub-group has the additional characteristic that more than 90% of the total alkaloid content in the plant species is the type VI alkaloid named lycopodine. Table 3 summarizes this information.

Table 3: Grouping of *Lycopodium* species based on alkaloid type

			Alkaloid Content	
			Feature Type	Other Types
Group 1		Urostachys	VIII	II, III, IV, V, VI
Group 2		Lepidotis	I or VII	III, IV, VI
Group 3	A.	Volubile, *L. deuterodensum*	VI	III, IV, V
	B.	Fastigiatum	VI	only IV
	C.	Complanata	VI >90% lycopodine	IV, V

Figure 2: Alkaloid skeletal types.



I: cernuane

II: lucidulane

III: phlegmarane

IV: fawcettidane

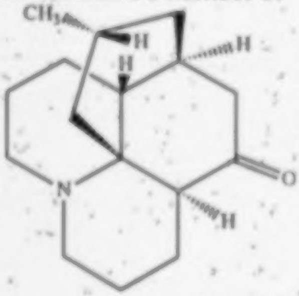
V: lycodane

VI: lycopodane

VII: inundatane

VIII: lucidane

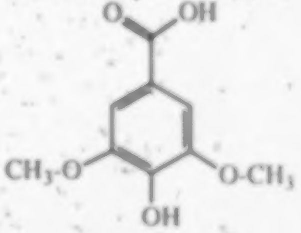
The alkaloid content of the *Lycopodium* plant species is useful for taxonomic studies, but it should be noted that Braekman's scheme is based only on approximately 10% of the known *Lycopodium* species. Furthermore, the scheme contains a number of discrepancies. For example in Braekman's scheme the species *L. inundatum* is placed in group 2. However, the Asian variety of *L. inundatum* contains alkaloids of a different skeleton type than the European variety (see Table 5). Although both varieties contain lycopodine, the difference in skeletal types of the other alkaloids suggests that these two plant varieties should not share the same species name. Another example was reported recently.



Lycopodine

Cernuine was isolated from *L. australianum*, a species classified in the *Urostachys* subgenus [20]. Cernuine was previously found only in species of the sub-genus *Lepidotis*. This could suggest that *L. australianum* really belongs in the *Lepidotis* subgenus, or that Braekman's scheme is not as well defined as it appears. In any case, much more work must be done in this area before any more conclusions can be drawn.

Other studies using the chemical content of the *Lycopodium* species as a taxonomic tool have been performed [22-24]. Twenty one species of *Lycopodium* were examined by Towers and Maass (1965) [24] for phenolic acids and for phenolic aldehydes, ketones and acids obtained upon ethanolysis or alkaline oxidation of their extracted woodmeals. The study found that syringic acid is present in species of *Lycopodium* only belonging to the sub-genera *Lycopodium* and *Diphasiastrum*. The *Lycopodium* species belonging to the sub genera *Huperzia* (*Urostachys*) or *Lycopodiella* (*Lepidotis*) do not contain detectable amounts of syringyl compounds.



Syringic Acid

Classification of the *Lycopodium* club mosses based on the alkaloid content might still prove to be a valuable taxonomic tool. A method for the rapid analysis of alkaloid content and identity has been developed for *Lycopodium* alkaloids by coupling gas chromatography with mass spectrometry (GC/MS), making it possible to examine many species in a short period of time. This has been shown to be a good method for the rapid screening of *Lycopodium* alkaloid extracts since, "the method is able to recognise new alkaloids as well as those of established structure and is applicable to small amounts of plant material [20]." Alkaloid extracts from a new plant species are injected into a gas chromatograph to separate the alkaloids. The exhaust port of the gas chromatograph is coupled to a mass spectrometer where the mass spectra of the separated alkaloids are collected and compared with the library spectra of alkaloids previously made up from a collection of known alkaloids.

B) The 'L' Numbered Alkaloids

In the first part of this thesis, GC/MS was used in conjunction with gas chromatography - infrared spectroscopy (GC/IR) to attempt to identify a number of previously isolated but unidentified alkaloid samples.

It was discovered as early as 1881 that alkaloids were present in *Lycopodium* [25]. However, it was not until the 1940's that these compounds were extensively investigated, and not until the late 1950's and early 1960's that the structures of these alkaloids began to be clarified. In Canada, the pioneering research on *Lycopodium* alkaloids was carried out by R.H.F. Manske and L. Marion, who isolated and characterized a large number of alkaloids from various species of club moss. They began their chemical investigation with the hypothesis that similarities of alkaloid constituents were indicative of botanical similarities and that this information could form the basis of a chemotaxonomic classification of the genus. Since that time the structures of about 100 alkaloids, classified into 16 structure types, have been established, and the alkaloid chemistry has been the subject of several reviews [26-28]. However, the structures of a number of alkaloids originally isolated by Manske and Marion remain unreported [28c]. Many of the alkaloids isolated by Manske and Marion were designated by numbers preceded by the letter L. In some cases an L numbered alkaloid isolate comprised a single compound (eg., L1, which has since been shown to be dihydrolycopodine), while in other cases, an L numbered alkaloid was shown to be a mixture (eg., L9, which was shown to be a mixture of lycopodine and acetyllofoline).

While the identity of many of these alkaloids has now been established, several remain unidentified. Many of the original *Lycopodium* samples of Manske and Marion were acquired by this group from Dr. O.E. Edwards, formerly of the National Research Council, Ottawa. Several of the L numbered alkaloids isolated over 40 years ago and whose structure or identity remained unresolved were found in their collection. The samples of alkaloids L15 (*L. tristachyum*), L16 and L17 (*L. obscurum*), L18

(*L. clavatum*), L22, L24, and L25 (*L. lucidulum*), L26 (*L. sabinaefolium*), L28, L29, and L31 (*L. annotinum*), and L35 (*L. densum*) were re-examined by GC-MS and GC-IR. Most of the samples proved to be mixtures of known alkaloids. The following table identifies the *Lycopodium* species examined by Manske and Marion containing as yet unidentified L numbered alkaloids.

Table 4: The unidentified 'L' numbered alkaloids of Marion and Manske.

Taxonomic Classification	**Unidentified Alkaloids**
Lycopodium	
L. annotinum L.	L10
L. annotinum var. *acrifolium*	L28, L29, L31
L. clavatum L.	L18
L. densum Labill.	L35
L. obscurum var. *dendroideum*	L16, L17
Diphasiastrum	
L. sabinaefolium Willd.	L26
L. tristachyum Pursh	L15
Huperzia	
L. lucidulum Michx.	L22, L24, L25

In total there were 35 alkaloids designated by L numbers by Manske and Marion. The structures of the majority of these alkaloids or alkaloid mixtures had been determined previously, but 13 remained unidentified. In the present work the structures of eleven of the remaining unidentified L numbered alkaloids have been determined. One alkaloid, L10, was not identified because a sample was unavailable. The structure of another alkaloid recently identified in this lab, L29, was confirmed by GC/IR and GC/MS in the present work. Part of this thesis describes the process of identification of some of the previously unidentified L numbered alkaloids.

C) *Lycopodium meridionale*

Lycopodium meridionale is a tropical species found in Central and South America. It has been observed growing in Jamaica [29], Puerto Rico [30], Cuba, Dominica, Guyana, and Brazil [17]. The plant material acquired for this research was collected in Guyana. *Lycopodium meridionale* was first described by Underwood and Lloyd in 1906. The name *'meridionale'* most likely comes from their description of this plant's habitat as being "probably widely distributed through the tropics of meridional America" [17]. At the time it was placed in the sub-genus *Diphasium* in the *Carolinianum* group. Since then much work has been carried out on the taxonomic classification of the *Lycopodium* club mosses and recently the *Carolinianum* group, including *L. meridionale,* has been placed in the sub-genus *Lepidotis* [12,16].

The alkaloids of *L. meridionale* have not been previously examined and part of the aim of the present work was to isolate and identify the alkaloids of this species. *Lycopodium meridionale* is closely related to *L. carolinianum* which is found in widely separated localities in America as well as in tropical Africa, the Mascarene Islands and tropical Asia [31]. A difference of opinion exists between the classification of this *Lycopodium* species as either *L. meridionale* or *L. carolinianum.* In fact, Nessel considered the species to be a variety of *L. carolinianum* (*L. carolinianum* var. *meridionale* (Underw. & Lloyd) Nessel [24]). The alkaloids of *L. carolinianum* have been examined previously as have the alkaloids of other *Lycopodium* species related to *L. meridionale,* in the sub-genus *Lepidotis* (Table 5).

The purpose of examining the alkaloids of *L. meridionale* was two-fold. First, it was to identify the alkaloids of this previously unexamined *Lycopodium* species with a view to clarifying its taxonomic classification. The second purpose was to look for new alkaloids that could contribute to the ongoing examination in this department of *Lycopodium* alkaloids as potential therapeutic agents for the treatment of Alzeihmer's Disease [32].

Table 5: *Lycopodium* alkaloids found in the sub-genus *Lepidotis*

L. carolinianum [33]
- anhydrolycocernuine
- carolinianine
- cernuine
- dihydrodesoxycernuine
- dihydrodesoxylycocernuine
- lycocernuine

L. cernuum [34-36c]
- cernuine
- dihydrodesoxycernuine
- lycocernuine
- N_{α}-methylphlegmarine

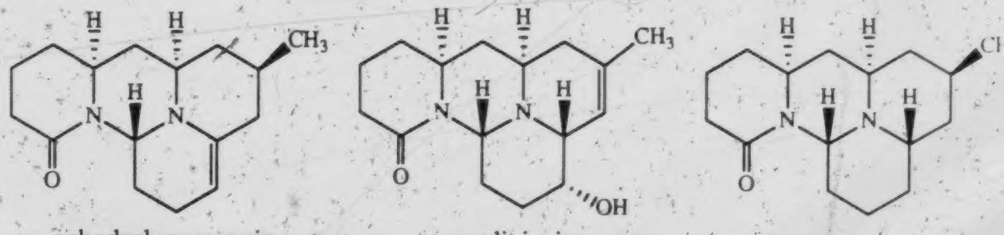
L. laterale [33]
- lycocernuine

L. inundatum (Asian variety) [37]
- anhydrolycocernuine
- lycocernuine
- lycopodine

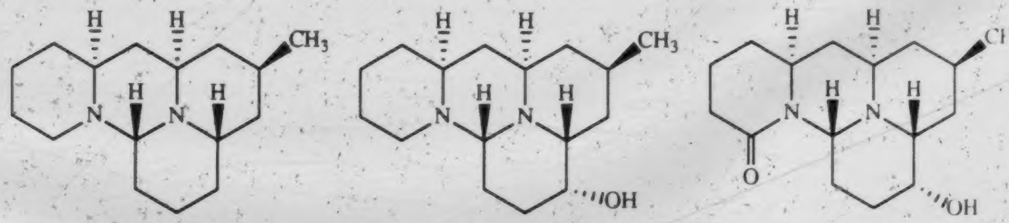
L. alopecuroides [36d-h]
- acetyldebenzoylalopecurine
- alolycopine
- alopecuridine
- alopecurine
- anhydrolycodoline
- clavolonine
- debenzoylalopecurine
- fawcettidine
- lycodoline
- lycopecurine
- lycpodine

L. inundatum (European variety) [38]
- dehydrolycopecurine
- inundatine
- isoinundatine
- lycodoline
- lycopodine

Figure 3: *Lycopodium* alkaloid structures from the sub-genus *Lepidotis*.

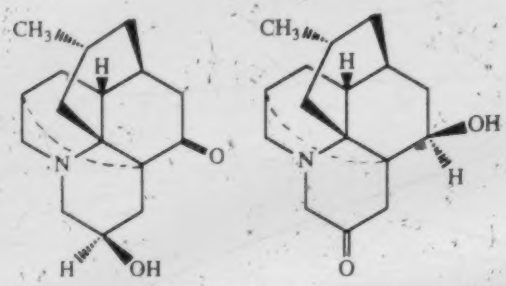


anhydrolycocernuine carolinianine cernuine

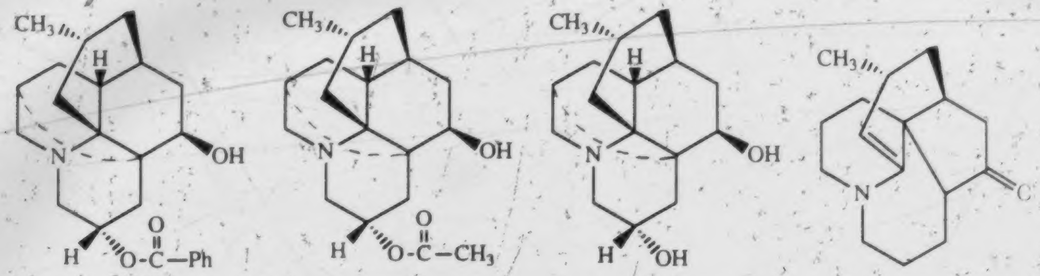


dihydrodesoxycernuine dihydrodesoxylycocernuine lycocernuine

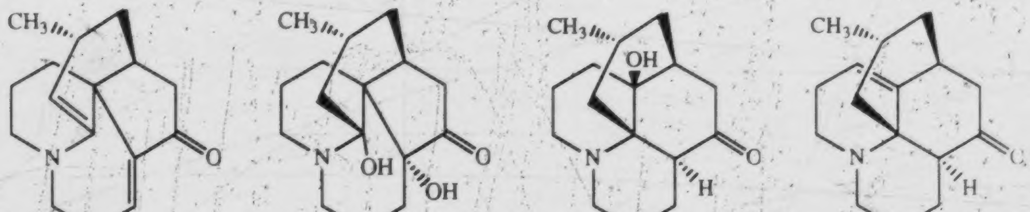
Figure 3 continued.



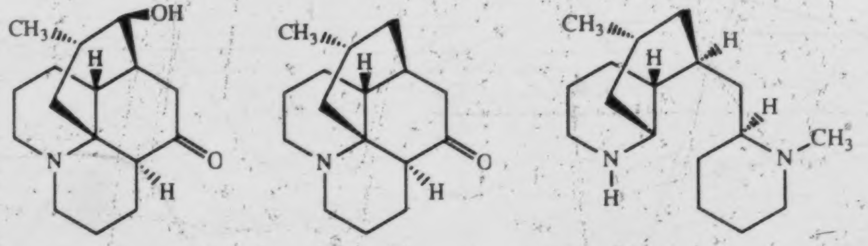
inundatine isoinundatine dehydrolycopecurine lycopecurine



alopecurine acetyldebenzoylalopecurine debenzoylalopecurine fawcettidine



alolycopine alopecuridine lycodoline anhydrolycodoline



clavolonine lycopodine N_α-methylphlegmarine

RESULTS AND DISCUSSION - The 'L' numbered Alkaloids*

The alkaloid identification was based on GC/MS [20] examination coupled with GC/IR [39] examination of the sample to rapidly establish the structure. A reference library of MS and IR spectra of known alkaloids was prepared from over 35 authentic samples available to us. In each GC/MS and GC/IR analysis a library search algorithm program was used to search the sample spectrum against the data base to find the best match to a sample spectrum. When the sample was found to be a mixture of alkaloids and sufficient sample was available, the components were separated and the identity of the alkaloids was verified by spectral analysis, including high resolution MS and NMR, and by comparison with authentic samples.

In the early 1940's Marion and Manske began their chemical investigation of the genus *Lycopodium* with the hypothesis that similarities in alkaloids were indicative of relationship and that this information could form the basis of a chemotaxonomic classification of the genus [42]. Accordingly a series of *Lycopodium* species were investigated, their alkaloids isolated, characterized, and designated with an L number [40-49].

In 1943, the first *Lycopodium* species, *Lycopodium complanatum* L. (later known as *L. flabelliformine* Fernald [42]), was investigated [40]. Examination of the alkaloids in this species led to the isolation of the L numbered alkaloids 1 to 6, as well as lycopodine and nicotine. The 'L' numbered alkaloids L1 - L6 were subsequently identified as dihydrolycopodine (L1) [50], O-acetyldihydrolycopodine (L2, L3) [51], anhydrodihydrolycopodine (L4) [52], flabellidine (L5) [53], and a mixture of α- and β-obscurine (obscurine, L6) [54].+

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* An account of this section has been published. Ayer, Browne, Elgersma & Singer 1990. Canadian Journal of Chemistry. 68: 1300-1304.

+ The structures of all the 'L' numbered alkaloids are collected in fig. 7, p 31-32.

In Braekman's scheme, *L. flabelliformine* falls into group C corresponding to *Lycopodium*, and in the third sub-group corresponding to *Complanata*. The alkaloids isolated from this plant species by Marion and Manske are of the skeletal types lycopodane (lycopodine, L1-L5) and lycodane (L6). This is consistent with Braekman's scheme.

In 1943, the alkaloids of *L. annotinum* L. were also investigated, leading to the isolation of L numbers 7 to 12 as well as lycopodine and α- and β-obscurine (L6 - obscurine). Some of these new L numbered alkaloids were later identified as annotinine (L7) [55], lycodoline (L8) [56], a mixture of lycopodine and acetyllofoline (L9) [57], annotine (L11) [58] and O-acetylacrifoline (L12) [59]. The structure of L10 has never been reported, and a sample of L10 was unavailable for the current investigation.

L. annotinum L. falls into group C corresponding to *Lycopodium* in Braekman's scheme and in the third sub-group corresponding to *Complanata*. The alkaloids isolated by Marion and Manske from this plant species are of the skeletal types lycopodane (lycopodine, L8, L9, L11, L12) and lycodane (L6), which is consistent with the taxonomic scheme proposed by Braekman. Annotinine (L7) is of a slightly different skeletal type than lycopodane, although it is appearently classified by Braekman as this type.

By 1944, the alkaloids of *L. tristachyum* Pursh had been examined by Marion and Manske. This led to the isolation of nicotine, lycopodine, L13, L14, and L15. L13 was later found to be identical with lycopodine [see Table 10] and L14 was subsequently identified as anhydrodihydrolycopodine [52]. L15 remained unidentified. Alkaloid L15 (reported as $C_{20}H_{31}NO_4$) [42] was analyzed by GC/MS and found to be composed of one major and one minor component with molecular ions of m/z 247 and 263, respectively. Analysis of the sample by GC/IR confirmed the presence of two components and identified lycopodine as the major alkaloid in the mixture. Since the components of the mixture eluted almost simultaneously from the GC column, it was not possible to unambiguously identify the minor alkaloid of L15. Thus alkaloid L15 was separated by chromatography. The least polar compound from the mixture was found to be identical with an authentic sample of lycopodine while the more polar compound was shown to be flabelliformine [60] by spectral analysis (^{1}H NMR, FTIR HREIMS) and by comparison with an authentic sample.

The compounds isolated from *L. tristachyum* by Marion and Manske are all of the skeletal type lycopodane. In Braekman's scheme, *L. tristachyum* falls under group C corresponding to *Lycopodium,* and in the third sub-group corresponding to *Complanata.* The alkaloid content of this species is consistent with this placement. However, this plant species could have been placed in any of the sub-groups of group C and still be consistent with Braekman's scheme.

In 1944, the alkaloids of *L. obscurum* var. *dendroideum* were examined by Manske and Marion [43]. This led to the isolation of lycopodine, L13, obscurine (L6), and two new alkaloids, L16 and L17. Alkaloid L16 ($C_{16}H_{25}NO$) was reported by these authors to be isomeric with lycopodine and L13. Analysis of alkaloid L16 by GC/MS revealed that it is a 1:1 mixture of two compounds with molecular ions of m/z 247 and 231. Subsequent examination of L16 by GC/IR confirmed the presence of two alkaloids and identified these as lycopodine and anhydrodihydrolycopodine. Examination of alkaloid L17 by GC/MS and GC/IR found it to be a 2:1 mixture of alkaloids. The minor component was identical with acetylacrifoline [59], and the structure of the major alkaloid was shown to be acetylannofoline [61].

In Braekman's scheme, *L. obscurum* var. *dendroideum* falls into group C, corresponding to *Lycopodium,* and in the first sub-group corresponding to *Volubile* and *L. deuterodensum.* The alkaloids isolated by Marion and Manske are of the skeletal type lycopodane (lycopodine (=L13), L16, L17) and lycodane (α- and β-obsurine - L6). This is consistent with Braekman's scheme.

Later in 1944, *L. clavatum* L. of North American origin was examined and found to contain nicotine, lycopodine, L13, L18, and L19 [44]. Alkaloid L13 has since been identified as lycopodine, and L19 was subsequently found to be identical with L34 and shown to be clavolonine [62,63]. Alkaloid L18 (reported as $C_{11}H_{19}NO$ and previously isolated as its picrate salt) was examined by GC/MS and found to contain two alkaloids (ratio 3:1) each with a molecular ion of 278 ($C_{18}H_{34}N_2$, HRMS) and very similar fragmentation patterns. GC/IR confirmed that alkaloid L18 was a mixture and that each of the component alkaloids contained N-methyl functionality (2780 cm^{-1}). Attempted separation of the sample by chromatography was unsuccessful. Analysis of the ^1H NMR spectrum of the mixture revealed the structural similarity of each component. Each component contains two N-methyl groups (δ 2.16, 2.28 (major); 2.25, 2.26 (minor)) and a secondary methyl (δ 0.84 (major); 0.92 (minor)). The spectral properties of the minor component are identical with that of N,N-dimethylphlegmarine[1] [64]. The major component is believed to be an isomer of this compound.

In Braekman's scheme *L. clavatum* L. falls into group C corresponding to *Lycopodium*, and in the first sub-group corresponding to *Volubile* and *L. deuterodensum*. The alkaloids isolated by Marion and Manske are of the skeletal types lycodane (lycopodine, clavolonine), and phlegmarane (N,N-dimethylphlegmarine, L18), and are in agreement with Braekman's scheme.

Because an old methanol extract of *L. clavatum* (variety unknown) was available, the alkaloids were extracted and screened by GC/IR for N,N-dimethylphlegmarine. This compound was not detected by this method. However, N_α-acetyl-N_β-methylphlegmarine, a compound of the same skeletal type has been isolated before from *L. clavatum* var. *borbonicum* [64]. The possibility that L18 is N,N-dimethylphlegmarine and an isomer is enhanced by this finding.

Marion and Manske reported on the examination of the alkaloids from *L. lucidulum* Michx. in 1946 [45]. Besides nicotine, lycopodine and L13, the L numbered alkaloids from L20 to L25 were isolated. Alkaloid L20 was later identified as 6α-

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[1] Special thanks to Professor J.C. Braekman for copies of the spectra of this compound.

hydroxyllycopodine [65], L21 later shown to be luciduline [66], and L23 subsequently found to be isolycodoline [67]. Alkaloid L22 was reported as $C_{16}H_{27}NO$, L24 reported as $C_{16}H_{25}NO$, and L25 reported as $C_{16}H_{25}NO_2$. Alkaloid L22 was examined by GC/MS and GC/IR. Each analysis revealed that it was a mixture of two alkaloids (ratio 3:2), one of which was lycopodine, while the other component was found to closely match the library spectra of isolycodoline. Separation of the sample by chromatography led to the isolation of lycopodine and isolycodoline, identical in all respects with authentic samples. Analysis of alkaloid L24 and L25 by GC/MS and GC/IR revealed that each was a single compound. Alkaloid L24 was identified as lycopodine while alkaloid L25 was found to be identical with isolycodoline (L23).

The species *L. lucidulum* Michx. is placed in group A corresponding to *Urostachys* by Braekman. The alkaloids isolated by Marion and Manske are of the skeletal types lycopodane (lycopodine (=L13), L20, L22, L23, L24, L25), and lucidulane (L21 luciduline), which is consistent with Braekman's scheme.

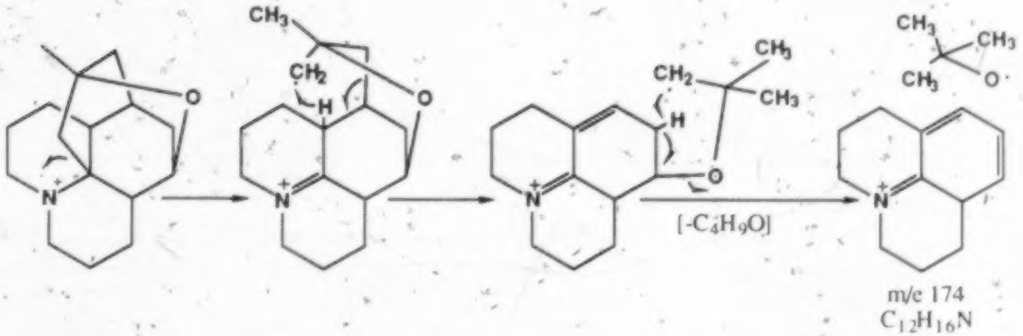
In a continuation of their studies in 1946, Manske and Marion reported that *L. sabinaefolium* Willd. contained four alkaloids: nicotine, lycopodine, alkaloid L13 and alkaloid L26 (reported as $C_{15}H_{25}NO$) [46]. Alkaloid L26 was analyzed by GC/MS and GC/IR, and was found to be a pure compound. The spectral data was matched with dihydrolycopodine [50] from the libraries of known alkaloids. Analysis of L26 by HREIMS showed a molecular formula of $C_{16}H_{27}NO$, in agreement with this match. Further analysis of the sample was not undertaken due to the small sample size.

The species *L. sabinaefolium* Willd. is placed by Braekman in group C corresponding to *Lycopodium*, and in sub-group 3 corresponding to *Complanata*. The alkaloids isolated by Marion and Manske from this species are all of the lycopodane skeletal type, consistent with Braekman's scheme. However, this plant species could have been placed in any of the sub-groups of group C and still be in agreement.

In 1947 it was reported that *L. annotinum* var. *acrifolium* contained a different alkaloid spectrum than other varieties of *L. annotinum*. In addition to lycopodine, annotinine, and acrifoline (L27), four previously unreported alkaloids were isolated and designated numbers L28 to L31 [47]. Subsequent investigations revealed that L30 was identical with L8, its structure was derived, and the compound was named lycodoline [55]. The structures of L28, L29, and L31 remain unreported to date. In earlier studies in these laboratories alkaloid L29 was identified as acrifoline by comparison (TLC, FTIR, HRMS) with an authentic sample [68,69].

Alkaloid L28 (reported as $C_{17}H_{27}NO_2$) was analyzed by GC/MS. It was shown to be a mixture of two compounds each of which has a molecular weight of 247 with similar but not identical mass spectral fragmentation patterns. Analysis by GC/IR confirmed the presence of two components and revealed that one of the components was lycopodine. The mixture was separated by chromatography over alumina. The least polar component, L28-1, was identified as lycopodine by comparison of its spectral properties with that of an authentic sample. The more polar component L28-2, was isolated as an optically active, air sensitive oil. The HRMS confirmed its molecular weight as 247 and established the molecular formula as $C_{16}H_{25}NO$. The similarity of the mass spectral fragmentation pattern in the MS of L28-2 and lycopodine suggested that they have the same carbon skeleton. The base peak at *m/z* 174 (M^{+}-C_4H_9O) is consistent with oxygen functionality of the "bridged atoms" (C-8, C-14, C15) [70] (Figure 4). Examination of the IR spectrum of L28-2 revealed the absence of hydroxyl or carbonyl functional groups and thus the oxygen of L28-2 could be present as an ether. This was corroborated by its ^{13}C NMR spectrum which showed oxygenated carbons at δ 74.3 (CH) and 72.3 (C). The ^{1}H NMR spectrum of L28-2 revealed a methyl singlet (δ 1.15) and this suggested that L28-2 may have the structure 5,15-oxidolycopodane. This compound has not been isolated previously from natural sources although it has been prepared synthetically in a series of reactions from lycopodine [71]. In order to confirm the identity of this alkaloid, dihydrolycopodine was treated with lead tetraacetate. The oxidation product obtained was identical in all respects (HRMS, FTIR, ^1H and ^{13}C NMR) with L28-2.

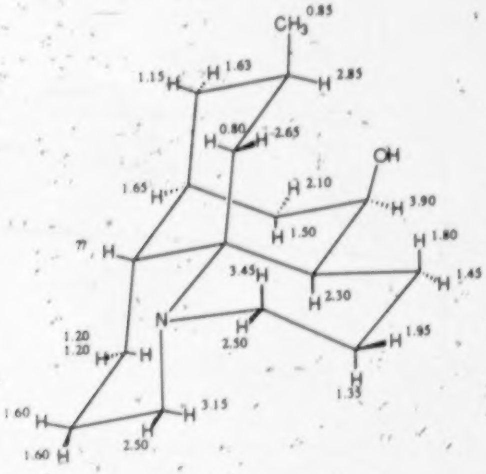
Figure 4: Mass fragmentation of the bridge of L28-2 (5,15-oxidolycopodane).



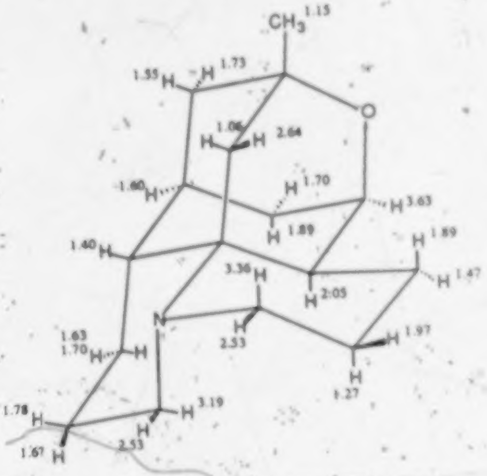
The complete ^1H and ^{13}C NMR assignments (Figures 5 and 6) have been made for 5,15-oxidolycopodane. The assignments were made based on the previous assignments for dihydrolycopodine [72] and O-acetyldihydrolycopodine [73] and from ^1H-^1H decoupling (Table 6), ^1H nOe [74] (Table 7), and ^1H-^1H COSY [75] spectra (Table 8) on 5,15-oxidolycopodane.

Another unidentified alkaloid isolated from *L. annotinum* var. *acrifolium*, L31 (reported as $C_{20}H_{29}NO_4$), was also found to be a mixture of two components, *m/z* 247 ($C_{16}H_{25}NO$) and *m/z* 359 ($C_{20}H_{31}NO_4$) by GC/MS. The presence of two components in L31 was confirmed by GC/IR analysis and the presence of 5,15-oxidolycopodane and acetyllofoline was suggested. Each component was separated by chromatography over alumina to give L31-1, identified as 5,15-oxidolycopodane by comparison with the spectral data obtained for L28-2, and L31-2, identified as acetyllofoline by comparison with an authentic sample [76].

Figure 5: ¹H NMR assignments for dihydrolycopodine and 5,15-oxidolycopodane.



Dihydrolycopodine

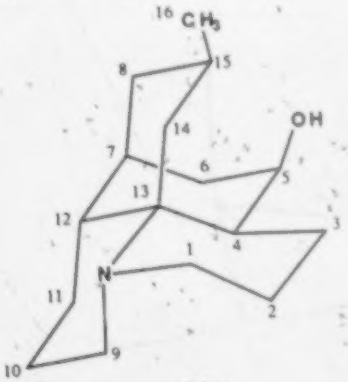


5,15-Oxidolycopodane

Figure 6: ¹³C NMR assignments for dihydrolycopodine and 5,15-oxidolycopodane.

Carbon number	Dihydro-lycopodine [72b]	Oxido-lycopodane
1	47.1	47.0
2	20.7	18.6
*3	23.5	24.6
4	32.6	31.4
5	68.5	74.3
6	34.0	31.0
7	35.6	33.9
8	42.0	42.1
9	47.4	47.4
*10	26.6	26.5
*11	25.0	25.6
12	45.8	46.8
13	55.2	54.3
14	43.2	43.9
15	23.5	72.3
16	24.1	29.2

* Assignments at C-3, C-10 and C-11 may be interchanged



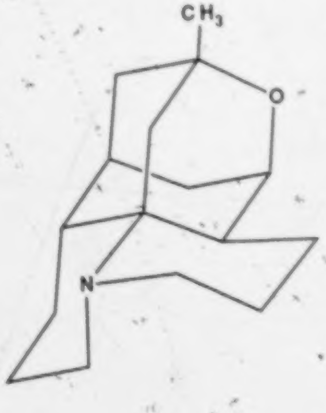


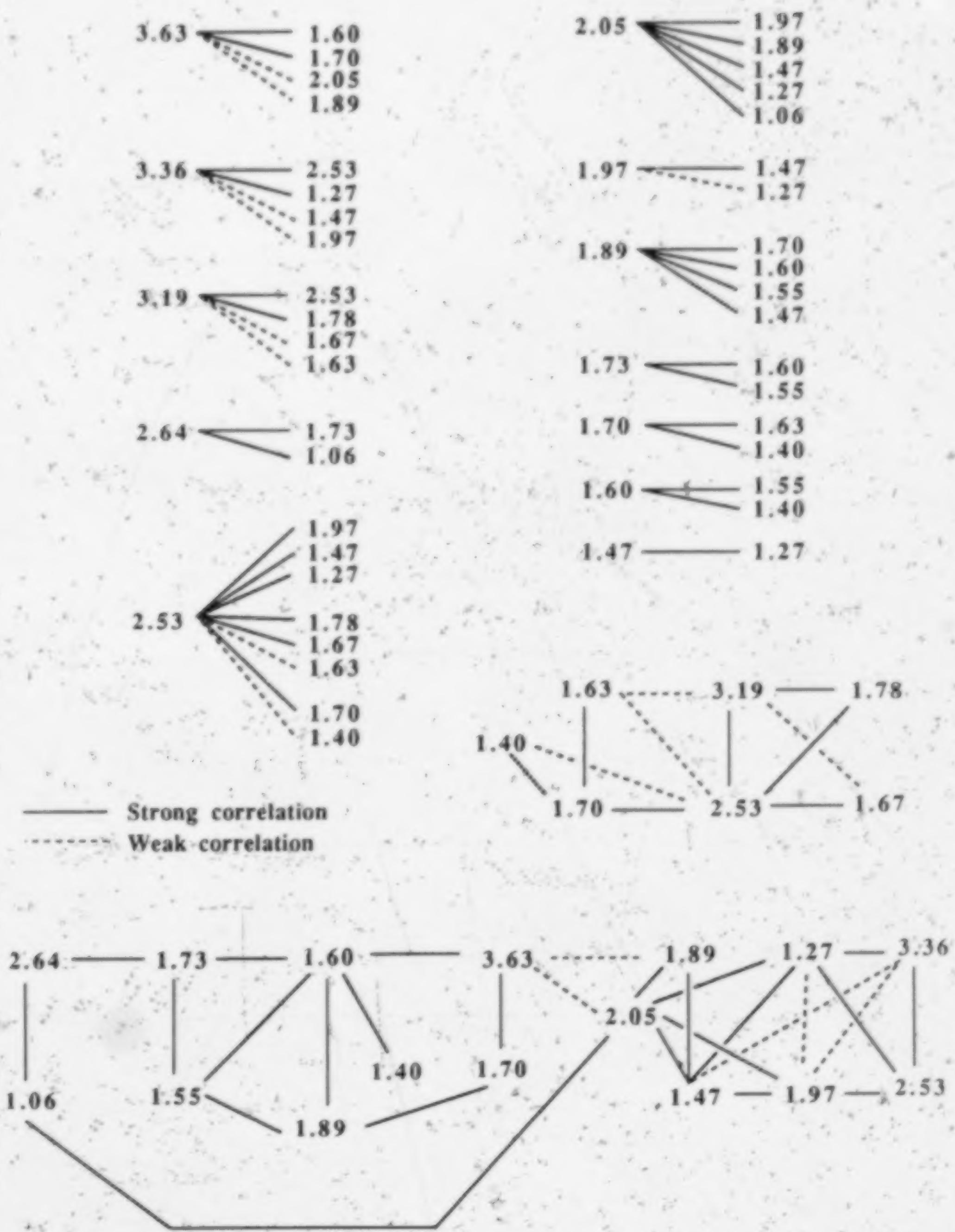
Table 6: ^{1}H NMR decoupling (CDCl$_3$) of 5,15-oxidolycopodane.

Signal irradiated (δ):		Observed change at (δ):		Coupling (Hz)
H_{1a}	3.36	H_{1e}	2.53	12.5
		H_{2a}	1.27	>3
H_{9a}	3.19	H_{9e}	2.53	12.5
H_{14e}	2.64	H_{8e}	1.73	2.0
		H_{14a}	1.06	12.0
$H_{1e,9e}$	2.53	H_{1a}	3.36	12.5
		H_{9a}	3.19	12.5
		H_{2e}	1.97	4.0
		H_{10e}	1.70	3.0
		H_{2a}	1.27	3.0
H_{14a}	1.06	H_{14e}	2.64	12.0

Table 7: ^{1}H NMR nOe (CDCl$_3$) of 5,15-oxidolycopodane.

Signal irradiated (δ):		nOe (%)	
H_5	3.63	H_4	2.05 (3.2)
H_{1a}	3.36	H_{14e}	2.64 (8.3)
		H_{1e}	2.53 (22.0)
H_{9a}	3.19	H_{9e}	2.53 (24.6)
		H_4	2.05 (5.8)
H_{14e}	2.64	H_{1a}	3.36 (9.2)
		H_{14a}	1.06 (15.5)
$H_{1e,9e}$	2.53	H_{1a}	3.36 (9.2)
		H_{9a}	3.19 (13.3)
H_{16}	1.15	H_{14e}	2.64 (7.5)
		H_{8e}	1.73 (4.2)
		H_{8a}	1.55 (1.7)

Table 8: ^{1}H NMR COSY (CDCl$_3$) of 5,15-oxidolycopodane.



The species *L. annotinum* var. *acrifolium* falls into group C corresponding to *Lycopodine,* and into the first sub-group corresponding to *Volubile* and *L. deuterodensum* in Braekman's scheme. The alkaloids isolated from this species by Marion and Manske are all of the lycopodane skeletal type. Marion and Manske have questioned the relationship between this species and *L. annotinum* L. in the past. "The absence of alkaloids L6, L8, L9, L11, and L12 all found in the type (*L. annotinum* L.) and the presence of 5 new ones (in *L. annotinum* var. *acrifolium*) indicates that this affinity can be only remote [47]." Indeed, even in Braekman's scheme, although these plant species have been placed in the same group and sub-group they could have been placed just as easily in separate sub-groups. The alkaloids of *L. annotinum* var. *acrifolium* are only of one skeletal type (lycopodane) (if annotinine can be classified as a lycopodane skeletal type), while the alkaloids of *L. annotinum* L. are of two types (lycopodane and lycodane).

In 1948, Marion and Manske examined the alkaloids from the tropical *species, L. cernuum* L., and three alkaloids, nicotine, cernuine (L32) and L33 were isolated [48]. The alkaloid lycopodine was conspicuously absent, and the new L numbered alkaloids appeared to contain a different carbon skeleton. The structures of cernuine and L33 (later named lycocernuine) were determined by others [35,36]. Both cernuine and lycocernuine were found to contain the same new and unique carbon skeleton.

In Braekman's scheme, L. *cernuum* is classified under group B corresponding to *Lepidotis.* The alkaloids isolated by Marion and Manske from this species are of the skeletal type cernuane, and are in agreement with Braekman's classification.

The last species of *Lycopodium* investigated by Marion and Manske was reported in 1953. Here, the alkaloids from *L. densum* Labill. *(L. deuterodensum* Herter), a species indigenous to New Zealand, were examined. From this plant, which has a lower than usual total alkaloid content, lycopodine, L34, and L35 were isolated [49]. L34 was subsequently identified as clavolonine [61, 62]. Alkaloid L35 was reported as $C_{14}H_{21}NO_2$. GC/MS and GC/IR analysis revealed that alkaloid L35 is a mixture of three components (ratio 5:1:2), and that these alkaloids are lycopodine, flabelliformine [60], and lycodoline (L8) [56], respectively. Alkaloid L35 was separated by chromatography. Comparison of the spectral data of each isolated compound with that of authentic samples confirmed their identity.

The name *L. densum* Labill. is synonymous with L. *deuterodensum* Herter. "*L. deuterodensum* Herter is more widely known as *L. densum* Labill., unfortunately an illegitimate later homonym of *L. densum* Lam. [77]." In Braekman's scheme, *L. deuterodensum* Herter falls into group C corresponding to *Lycopodium*, and in the first sub-group corresponding to *Volubile* and *L. deuterodensum*. The alkaloids isolated by Marion and Manske from this species are all of the skeletal type lycopodane, and is consistent with Braekman's scheme. However, this species could have been placed in any of the sub-groups of group C and still be consistent.

Had Marion and Manske known the structures of all the alkaloids they isolated, they might have started a taxonomic classification scheme based on alkaloid skeletal types. Two very distinct classes are observed, namely plants containing alkaloids of the lycopodine type and those containing alkaloids of the cernuane class [Table 9]. Within the species that contain the lycopodine type alkaloids, four sub-classes could be formed: a) those plants containing alkaloids only of the lycopodane type, b) those containing lycodane type alkaloids in addition to lycopodane type, c) those containing lucidane type alkaloids in addition to lycopodane type, and d) those containing phlegmarane type alkaloids in addition to lycopodane type alkaloids.

In many of the *Lycopodium* species examined by Marion and Manske, the alkaloid nicotine was isolated. Nicotine has been isolated from species of plants other than *Lycopodium*, and its presence is probably not taxonomically useful.

Table 9: Alkaloid skeletal types present in the *Lycopodium* species examined by Marion and Manske.

Lycopodium species examined by Marion and Manske	Alkaloid skeletal types
L. annotinum var. *arcrifolium*	lycopodane
L. densum Labill. (*L. deuterodensum* Herter)	lycopodane
L. tristachyum Pursh	lycopodane
L. sabinaefolium Willd.	lycopodane
L. annotinum L.	lycopodane lycodane
L. complanatum L. (*L. flabelliforme*)	lycopodane lycodane
L. obscurum var. *dendroideum*	lycopodane lycodane
L. lucidulum Michx.	lycopodane lucidulane
L. clavatum L.	lycopodane phlegmarane
L. cernuum L.	cernuane

The alkaloids isolated from the *Lycopodium* species have proved to be a rich source of chemically interesting compounds. In total 35 alkaloids were designated with a L number. The structures or identity of the majority of these alkaloids have been determined by others. The known alkaloids together with the previously unidentified alkaloids are summarized in Table 10. They are presented in order of increasing L number, and the table includes their molecular weight, molecular formula, name, and reference to their structure determination. The structures of the L numbered alkaloids are given in figure 7.

Table 10: The 'L' Numbered *Lycopodium* Alkaloids.

L#	Molecular weight	Molecular formula	Name [Reference]
L1	249	$C_{16}H_{27}NO$	dihydrolycopodine (complanatine, L26)[50]
L2	291	$C_{18}H_{31}NO_2$	O-acetyldihydrolycopodine [51]
L3	291	$C_{18}H_{31}NO_2$	O-acetyldihydrolycopodine [51]
L4	231	$C_{16}H_{25}N$	anhydrodihydrolycopodine [52][1]
L5	288	$C_{18}H_{28}N_2O$	flabellidine [53]
L6			α- and β-obscurine [54]
L7	275	$C_{16}H_{21}NO_3$	annotinine [55]
L8	263	$C_{16}H_{25}NO_2$	lycodoline (L30) [56]
L9			lycopodine and O-acetylflofoline [57, 76]
L10			structure undetermined and sample unavailable
L11	288	$C_{16}H_{21}NO_3$	annotine [58]
L12	305	$C_{18}H_{25}NO_3$	O-acetylacrifoline [59]
L13	247	$C_{16}H_{25}NO$	lycopodine[2]
L14	231	$C_{16}H_{25}N$	anhydrodihydrolycopodine [52][1]
L15*			lycopodine and flabelliformine [60]
L16*			lycopodine and anhydrodihydrolycopodine [52]
L17*		$C_{18}H_{27}NO_3$	acetylannofoline [61] and acetylacrifoline [58]
L18*			N,N-dimethylphlegmarine and isomer
L19	263	$C_{16}H_{25}NO_2$	(L34), clavolonine [62, 63]
L20	263	$C_{16}H_{25}NO_2$	6α-hydroxylycopodine [65]
L21	207	$C_{13}H_{21}NO$	luciduline [66]
L22*			lycopodine and isolycodoline (L23) [67]
L23	263	$C_{16}H_{25}NO_2$	isolycodoline (epilycodoline, pseudoselagine) [67]
L24*	247	$C_{16}H_{25}NO$	lycopodine

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* Alkaloids identified in this or other studies in our laboratories.

[1] L4 is identical with L14, identified as anhydrodihydrolycopodine by Marion and Manske (laboratory notebook 698, 15). The identities of L4 and L14 were confirmed by GC/MS and GC/IR.

[2] L13 was identified as lycopodine by Marion and Manske (laboratory notebook 698, 57). Examination of eleven samples of L13,isolated from several *Lycopodium* species, by GC/MS and CG/IR found them to be comprised mainly of lycopodine.

Table 10 continued...

L25*	263	$C_{16}H_{25}NO_2$	isolycodoline (epilycodoline, pseudoselagine) [67]
L26*	249	$C_{16}H_{27}NO$	dihydrolycopodine (L1) [50]
L27	261	$C_{16}H_{23}NO_2$	acrifoline [68, 69]
L28*			lycopodine and oxidolycopodane
L29*	261	$C_{16}H_{23}NO_2$	acrifoline [68, 69][3]
L30	263	$C_{16}H_{25}NO_2$	lycodoline (L8) [56]
L31*			O-acetyllofoline [76] and oxidolycopodane
L32	262	$C_{16}H_{26}N_2O$	cernuine [35,36]
L33	278	$C_{16}H_{26}N_2O_2$	lycocernuine [35,36]
L34	263	$C_{16}H_{25}NO_2$	clavolonine (L19) [62, 63]
L35*			lycopodine, flabelliformine [9], and lycodoline [24]

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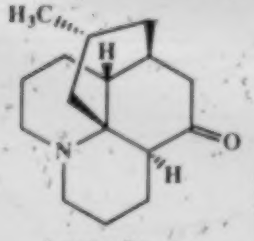
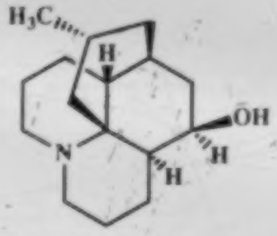
[3] Identified by T. Chua, Alberta Heritage Undergraduate Summer Research Assistant, 1986.

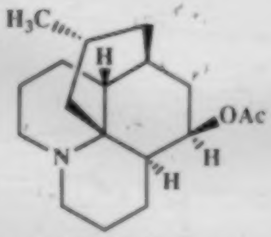
Figure 7: Structures of the 'L' numbered alkaloids.



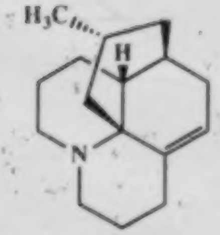
Lycopodine



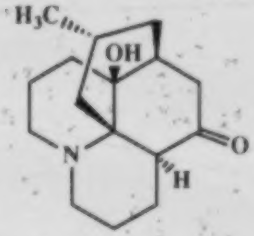
Dihydrolycopodine



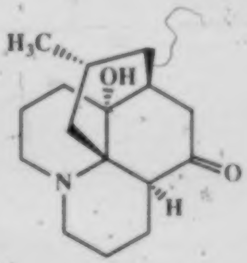
O-acetyldihydrolycopodine



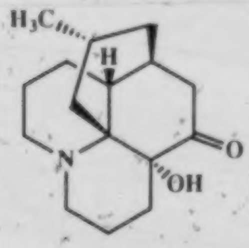
Anhydrodihydrolycopodine



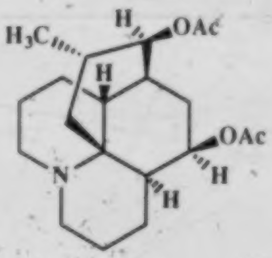
Lycodoline



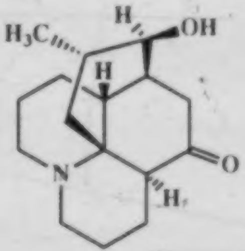
Isolycodoline



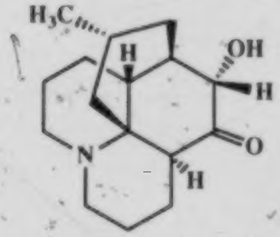
Flabelliformine



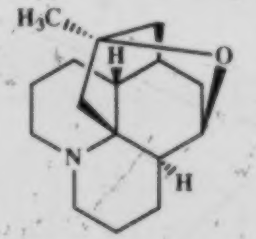
O-acetyllofoline



Clavolonine



6α-Hydroxylycopodine



Oxidolycopodane

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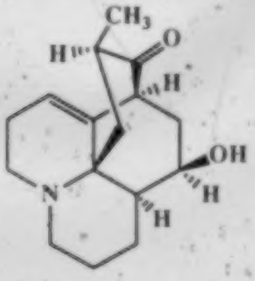
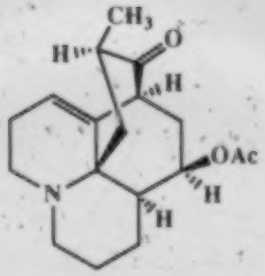
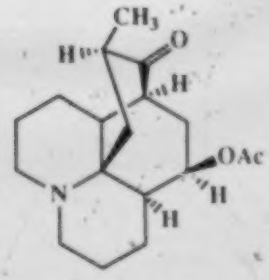
Figure 7 continued...



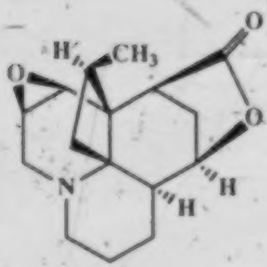
Acrifoline



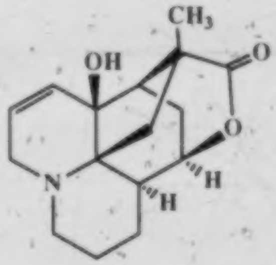
O-acetylacrifoline



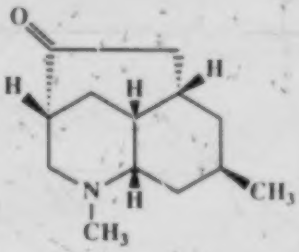
O-acetylannofoline



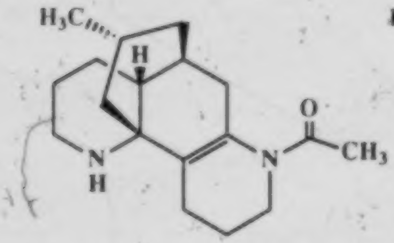
Annotinine



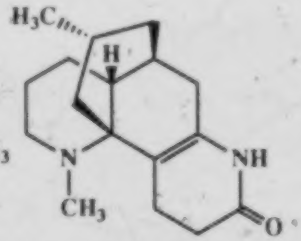
Annotine



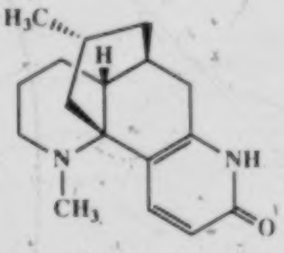
Luciduline



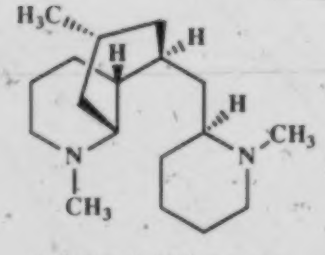
Flabellidine



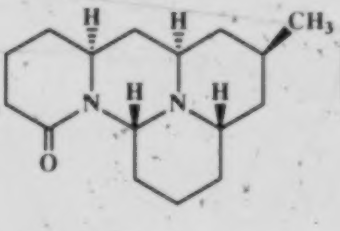
α-obscurine



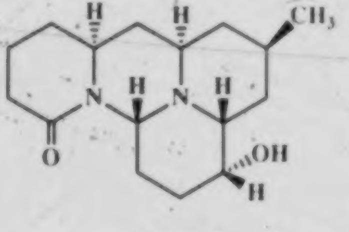
β-obscurine



N,N-dimethylphlegmarine



Cernuine



Lycocernuine

The 'L' Numbered Alkaloids

RESULTS AND DISCUSSION - *Lycopodium meridionale*

The alkaloid constuents of *Lycopodium meridionale* were isolated according to the procedure of Burnell [78], and purified by chromatography over alumina (see experimental). In this manner, three alkaloids (LM-1, LM-2 and LM-3) were obtained in pure form. One of the compounds (LM-2) was identified as lycocernuine by comparison of spectral data with that of an authentic sample.

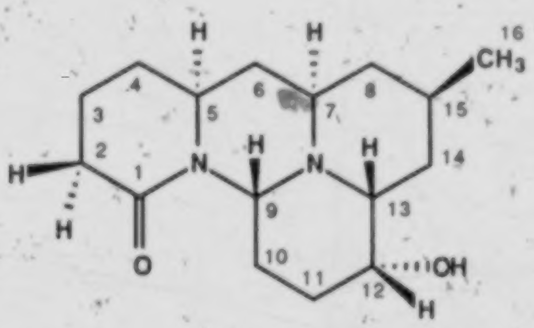
Identification of LM-1.

LM-1 was isolated as a colorless oil that crystallized upon standing as prisms, and melted at 89-92°C. The molecular formula was determined from the high resolution mass spectrum as $C_{16}H_{24}N_2O_2$ and confirmed by a chemical ionization (NH_3) mass spectrum which shows an ion at *m/z* 277 (100%) corresponding to M+H. The infrared spectrum of LM-1 shows a strong band at 1640 cm^{-1} ($CHCl_3$ cast) suggesting the presence of a lactam as in cernuine and lycocernuine which both show a strong absorption at 1640 cm^{-1} (CCl_4 sol.) [35]. The presence of a lactam group was confirmed by a signal at δ 168.8 in the ^{13}C NMR spectrum of LM-1. The 15 remaining signals in the ^{13}C NMR spectrum of LM-1 occur upfield of δ 80.8 hence the second oxygen atom in LM-1 must be present as either an ether or a hydroxyl group. The absence of any bands above 3000 cm^{-1} in both the $CHCl_3$ cast and solution IR spectra, and no observed deuterium exchange in the ^1H NMR spectrum suggests that there is no hydroxyl group present. Attempted acetylation of LM-1 with acetic anhydride in pyridine containing a catalytic amount of dimethylaminopyridine failed. Thus, the second oxygen atom can be accounted for if an ether is present in LM-1. In the ^{13}C spectrum of LM-1, deshielded carbon resonances are observed at δ 80.8 ppm (C) and δ 74.8 ppm (CH), and these can be assigned to oxygen bearing carbon atoms.

From the molecular formula, $C_{16}H_{24}N_2O_2$, there are 6 sites of unsaturation in the molecule, and one of these can be accounted for by the amide carbonyl. Since there is no spectral evidence to suggest the presence of either double or triple bonds in LM-1, the five remaining sites of unsaturation indicated by the molecular formula can be accounted for if LM-1 is pentacyclic.

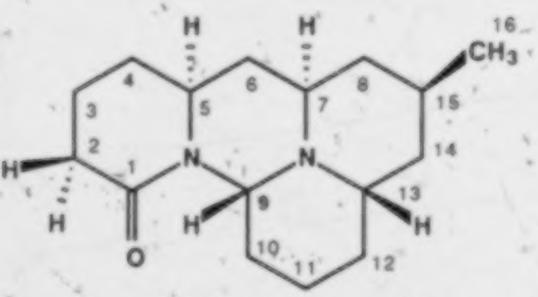
Further evidence for the structure of LM-1 was obtained from a comparison with the ^{1}H and ^{13}C NMR of lycocernuine and cernuine. The ^{1}H NMR spectra of lycocernuine and cernuine were previously recorded using 60 and 100 MHz instruments, and the ^{13}C NMR spectra for these compounds were not reported previously. In the present investigation, the spectra of lycocernuine and cernuine were examined using a 360 MHz NMR instrument, thereby allowing better resolution of the NMR spectra (Tables 11, 12, and 13). A sample of cernuine was obtained from the collection of *Lycopodium* alkaloids available in this laboratory. A comparison of the spectral data of lycocernuine with that of LM-1 (Table 14), suggests a structural similarity between these compounds.

Table 11: ^{1}H NMR spectral data for Lycocernuine ($CDCl_3$).



Position	Literature (100 Mhz) [33]	Observed
2a		2.41, dddd, J = 17, 5.5, 4.0, 2.0 Hz
2b		2.31, dd, J = 17, 11, 5.5 Hz
5	3.46, m	3.49, m
7	3.46, m	3.49, m
9	5.44, dd, J = 12, 2 Hz	5.43, dd, J = 12, 2.5 Hz
12	3.73, dd, J = 2, 2 Hz	3.79, ddd, J = 5.0, 2.5, 2.5 Hz
13	2.92, dd, J = 6, 2 Hz	2.94, dm, J = 6.5 Hz
15	0.85, d, J = 6 Hz	0.85, d, 6.5 Hz

Table 12: ^1H NMR spectral data for Cernuine ($CDCl_3$).



Position	Literature (60 Mhz) [36a]	Observed
2a		2.36, dddd, J = 17, 5.5, 4.0, 2.0 Hz
2b		2.26, ddd, J = 17, 11, 6.0 Hz
5	2.9-3.7	3.07, m
7	2.9-3.7	3.04, m
9	5.47, dd, J = 11, 2.5 Hz	5.43, dd, J = 11.5, 3.0 Hz
13	2.9-3.7	3.44, dddd, J = 11, 9.0, 5.0, 3.0 Hz
15	0.86, d, J = 6 Hz	0.82, d, J = 6.5 Hz

Table 13: ^{13}C Spectral data for cernuine and lycocernuine ($CDCl_3$).*

Carbon Number	Cernuine	Lycocernuine
1	168.1	168.4
2	39.3	38.0
3	33.0	33.2
4	30.5	30.5
5	50.6	51.0
6	20.0	19.3
7	57.5	58.6
8	41.2	41.8
9	67.2	67.3
10	19.3	15.9
11	24.5	33.8
12	22.3	71.0
13	46.1	49.0
14	42.0	41.8
15	25.3	26.4
16	22.2	23.0

*Assignments are tentative.

Table 14: Spectral comparisons between LM-1 and lycocernuine.

LM-1		**LM-2 (lycocernuine)**	
^{1}H NMR: In chloroform		**^{1}H NMR:** In chloroform	
5.54 (1H, dd, 10, 5.0 Hz)		5.43 (1H, dd, 12, 2.5 Hz)	
3.99 (1H, ddd, 5, 2.0, 2.0 Hz)		3.79 (1H, ddd, 5, 2.5, 2.5 Hz)	
3.58 (2H,m)		3.49 (2H, m)	
3.32 (1H, dd, 3.0 , 3.0 Hz)		2.94 (1H, dm, 6.5 Hz)	
2.44 (1H,dddd, 16, 4.0 , 2.5, 2.5 Hz)		2.41 (1H, dddd, 17, 5.5, 4.0, 2.0 Hz)	
2.35 (1H, ddd, 16, 11, 6.0 Hz)		2.31 (1H, dd, 17, 11, 5.5 Hz)	
2.27 (2H, m)		2.25 (2H, m)	
1.34 (3H, s)		0.85 (3H, d, 6.5 Hz)	
In benzene		In benzene	
5.77 (1H, dd, 11.0, 5.0 Hz)		5.75 (1H, dd, 12, 2.5 Hz)	
3.63 (1H, ddd, 5.0, 2.0, 2.0 Hz)		3.36 (1H, dddd, 6.0, 6.0, 3.0, 3.0 Hz)	
3.36 (1H, dddd, 11, 11, 6, 2 Hz)		3.14 (1H, ddd, 5.0, 2.5, 2.5 Hz)	
2.99 (1H,m)		3.06 (1H, m)	
2.85 (1H, dd, 2.0, 2.0 Hz)		2.60 (1H, dm, 6.5 Hz)	
2.36 (1H, dddd, 17, 5, 2.5, 2.5 Hz)		2.34 (1H, dddd, 17, 5.5, 4, 2 Hz)	
2.15-1.90 (3H, m)		2.30 (1H, m)	
1.24 (3H, s)		2.15 (1H,dd, 17, 11, 5.5 Hz)	
		0.89 (3H, d, 6.5 Hz)	
^{13}C NMR: In chloroform, APT		**^{13}C NMR:** In chloroform, APT	
168.8 (C)	74.8 (CH)	168.4 (C)	71.0 (CH)
80.8 (C)	66.3 (CH)	41.8 (CH$_2$)	67.3 (CH)
44.3 (CH$_2$)	62.3 (CH)	41.8 (CH$_2$)	58.6 (CH)
43.1 (CH$_2$)	53.0 (CH)	38.0 (CH$_2$)	51.0 (CH)
39.1 (CH$_2$)	48.4 (CH)	33.8 (CH$_2$)	49.0 (CH)
32.9 (CH$_2$)	25.7 (CH$_3$)	33.2 (CH$_2$)	**26.4 (CH)**
30.8 (CH$_2$)		30.5 (CH$_2$)	23.0 (CH$_3$)
24.9 (CH$_2$)		19.3 (CH$_2$)	
20.0 (CH$_2$)		15.9 (CH$_2$)	
13.9 (CH$_2$)			

The conformation of lycocernuine was determined previously by Ayer et al.[36b] in the following way. The equatorial nature of the hydrogen geminal to the hydroxyl group at C_{12} (and hence the axial nature of the hydroxyl group) was demonstrated by the small couplings (2.1-2.5 Hz) of this hydrogen to hydrogens at C_{11} and C_{13}. The shape of the hydrogen signal at C_9 (dd, 11, 2 Hz) indicates that it is an axial hydrogen, and is consistant with the assignment of the configuration at C_9. As well, no evidence for intramolecular hydrogen bonding between the hydroxyl group and the basic nitrogen atom was observed in the infrared spectrum. This suggests that the hydroxyl group is remote from the lone pair of electrons on the basic nitrogen atom.

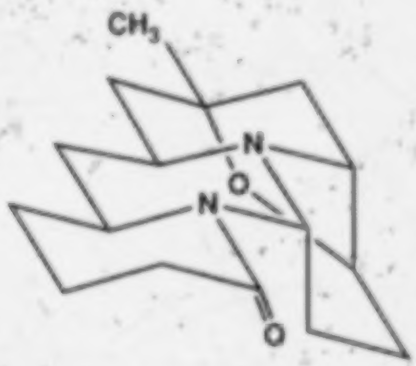
In the ^{1}H NMR spectrum for LM-1, small couplings are observed (5.0, 2.0 Hz) between the hydrogen at C_{12} and those on C_{11} and the one on C_{13}, and the signal for the hydrogen at C_9 of LM-1 shows couplings of 10 and 5.0 Hertz. This indicates the equatorial nature of the hydrogen at C_{12} and the axial nature of the hydrogen at C_9 as in lycocernuine. Also, the chemical shifts and shapes of the multiplets attributed to C_5 and C_7 for lycocernuine are similar to those observed for LM-1, and show similar solvent induced shifts (Table 15) going from chloroform to benzene. If the configuration at these two carbon atoms was different for these two compounds, this would not be expected.

Table 15: ^{1}H NMR Solvent induced shifts in lycocernuine and LM-1.

Position	Lycocernuine δ ($CDCl_3$) - δ (Benzene d6) [ppm]	LM-1 δ ($CDCl_3$) - δ (Benzene d6) [ppm]
2a	0.16	0.22
2b	0.07	0.08
5	0.43	0.59
7	0.13	0.22
9	-0.32	-0.23
12	0.65	0.36
13	0.34	0.47
16	-0.04	0.10

The position of the ether linkage of LM-1 was determined in the following manner. In the ^{1}H NMR spectrum of LM-1 the C_{16} methyl hydrogens occur as a singlet at δ 1.34 whereas the C_{16} methyl hydrogens occur as a doublet (6.5 Hz) at δ 0.85 in the spectrum of lycocernuine. This suggests an oxygen functionality at C_{15} for LM-1. The presence of an ether linkage between C_{12} and C_{15} is apparent from an examination of the ^{13}C NMR of LM-1. Thus, the position of the C_{12} signal in the spectrum of LM-1 (δ 74.8) is similar to that of C_{12} in lycocernuine (δ 71.0). However, the position of the C_{15} signal for LM-1 (δ 80.8) is very much further downfield than the corresponding signal in lycocernuine (δ 26.4) as might be expected if there was a C_{12}-C_{15} ether linkage in LM-1. As well, the C_{16} methyl signal of LM-1 (δ 25.7) is further downfield than the corresponding signal in lycocerniune (δ 23.0).This suggests the following structure for LM-1.

Figure 8: The proposed structure for LM-1.



The mass spectrum supports the proposed structure of LM-1, and is similar to that observed for lycocernuine (Table 16). An important difference between the two spectra is an apparent similarity. An ion at *m/z* 261 is observed in both spectra, however the formation of this ion, $[C_{16}H_{25}N_2O]^+$, from lycocernuine is by loss of the hydroxyl group, whereas the formation of this ion, $[C_{16}H_{21}N_2O_2]^+$, from LM-1 is by loss of a methyl group (the high resolution mass spectra confirm the different ionic formulas). The pronounced loss of the methyl group from LM-1 is another indication of a C_{12}-C_{15} ether linkage.

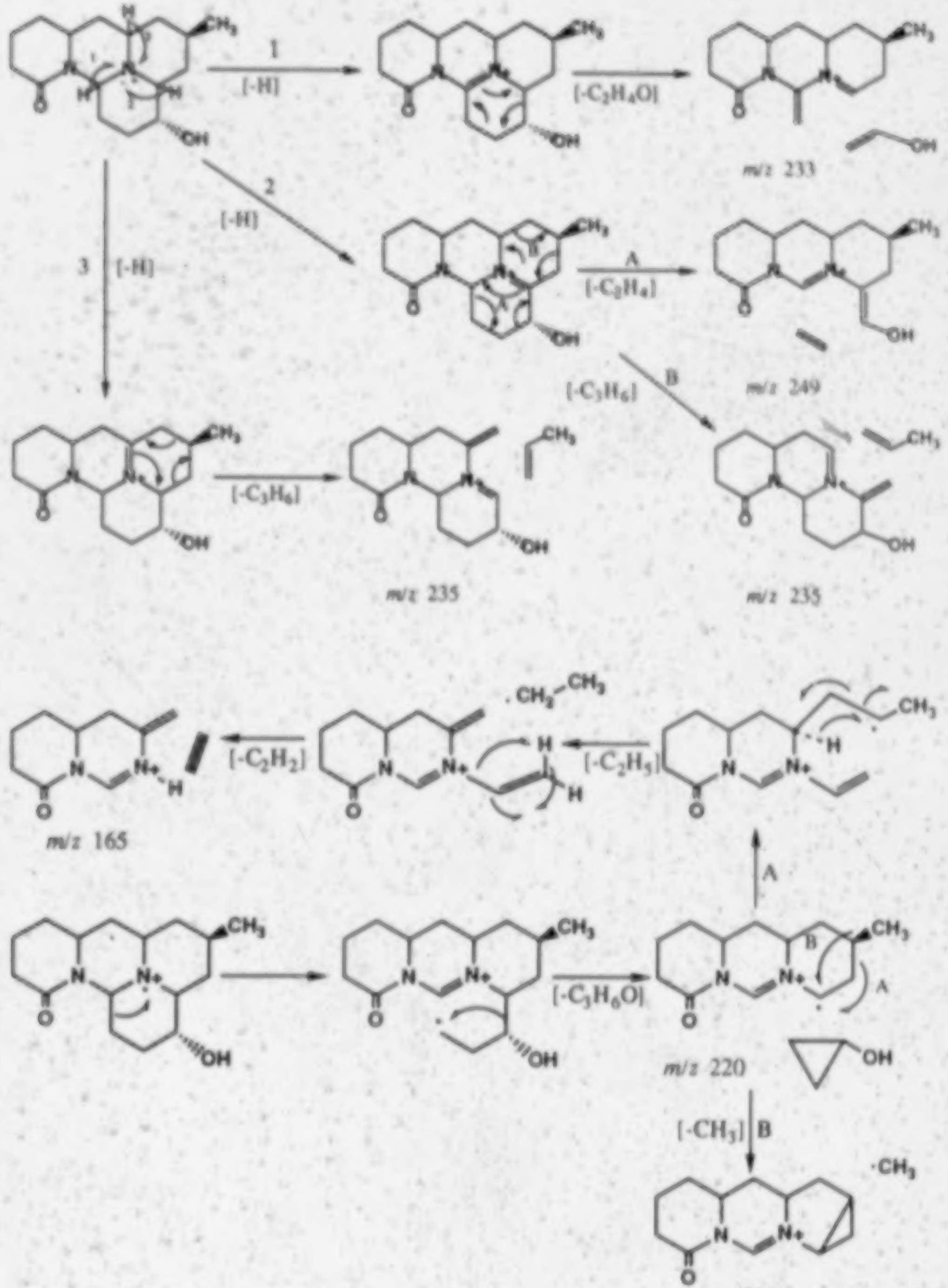
Table 16: Mass spectral data for LM-1 and lycocernuine (HREIMS).

LM-1			**Lycocernuine**		
M/z (%)	Formula	Loss of ...	M/z (%)	Formula	Loss of ...
276 (100)	$C_{16}H_{24}N_2O_2$	•	278 (50)	$C_{16}H_{26}N_2O_2$	•
261 (33)	$C_{16}H_{21}N_2O_2$	CH_3	261 (16)	$C_{16}H_{25}N_2O$	OH
247 (4.2)	$C_{14}H_{19}N_2O_2$	C_2H_5	249 (22)	$C_{14}H_{21}N_2O_2$	C_2H_5
233 (47)	$C_{14}N_{21}N_2O$	C_2H_3O	235 (4.0)	$C_{13}H_{19}N_2O_2$	C_3H_7
219 (11)	$C_{13}H_{19}N_2O$	C_3H_5O	233 (3.1)	$C_{14}H_{21}N_2O$	C_2H_5O
209 (38)	$C_{11}H_{17}N_2O_2$	C_5H_7	219 (100)	$C_{13}H_{19}N_2O$	C_3H_7O
205 (13)	$C_{12}H_{17}N_2O$	C_4H_7O	205 (4.5)	$C_{12}H_{17}N_2O$	C_4H_9O
165 (47)	$C_9H_{13}N_2O$	$C_7H_{11}O$	165 (23)	$C_9H_{13}N_2O$	$C_7H_{13}O$

Schemes 1 and 2 summarize some possible fragmentation pathways for lycocernuine [35,79] and LM-1. However, more than one fragmentation pathway can be responsible for the same observed peaks in the mass spectrum of lycocernuine, as was demonstrated using deuterium isotopes of lycocernuine [79], and hence the fragmentations are more complex than shown in Scheme 1. The fragmentation pathways for LM-1 could also then be more complex than shown Scheme 2. The fragmention pathways proposed for LM-1 were based on observed pathways for lycocernuine and cernuine, and on known fragmentations of ethers [80].

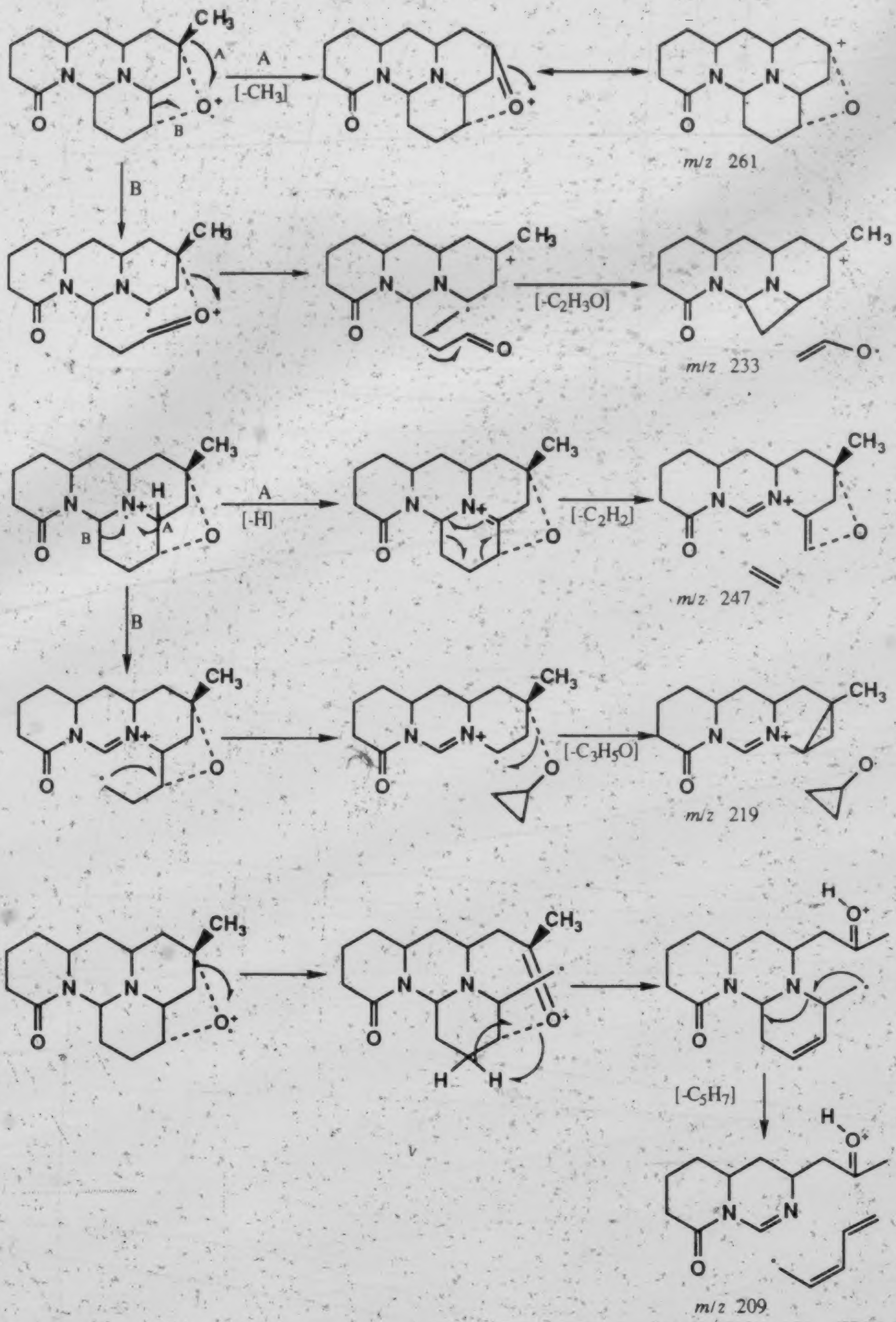
Most of the fragmentation pathways for lycocernuine are consistant with radical initation at one of the nitrogen atoms, although the loss of the hydroxyl group is an exception. It appears from examination of the mass spectral data of LM-1 that there is more tendancy for radical initiation at the oxygen atom since, for example, this would explain the loss of the methyl group to form the *m/z* 261 ion and the loss of C_5H_7 to form the *m/z* 209 ion in LM-1.

Scheme 1: Mass fragmentation pathways for lycocernuine.



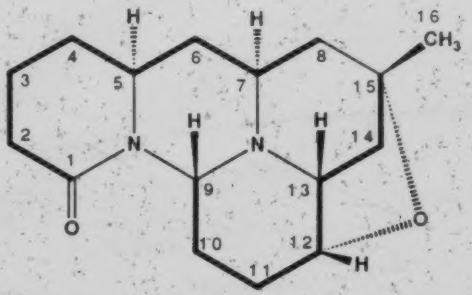
Lycopodium meridionale

Scheme 2: Mass fragmentation pathways for LM-1.



To further confirm the proposed structure of LM-1 and to complete the ^{1}H NMR assignments, ^{1}H nOe (Table 17), ^{1}H-^{1}H decoupling (Table 18), and ^{1}H-^{1}H shift correlated 2D NMR (COSY 90) (Figures 12,13) spectra were obtained. The ^{1}H NMR assignment of LM-1 (Figure 11) was based on comparison of the spectral data with lycocernuine (obtained in deuterobenzene), as well as additional data obtained for LM-1. The assignments for LM-1 are based on data aquired in deuterobenzene as the solvent since the signals for the hydrogens at C_5 and C_7 are completely separated and other regions of the spectrum are clearer in this solvent. From a comparison with lycocernuine the following signals can be assigned for LM-1. The signal at δ 5.77 can be assigned to the hydrogen at C_9, the signal at δ 3.63 to the hydrogen at C_{12}, the signal at δ 2.85 to the hydrogen at C_{13}, and the signals at δ 2,99 and δ 3.36 to hydrogens at C_5 and C_7 (relative assignment of these two signals unknown at this point). As well, the signal at δ 1.24 can be assigned to the methyl group (C_{16}), and the signals at δ 2.36 and δ 2.13 to the hydrogens on C_2.

Figure 9: Numbering scheme for LM-1.

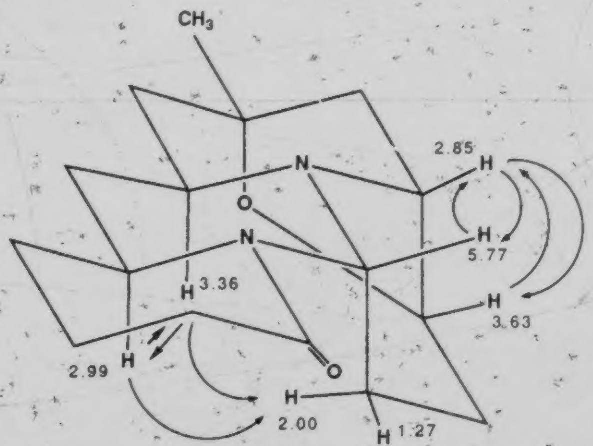


The hydrogens corresponding to the signals at δ 5.77 and δ 2.85 are in axial positions based on the proposed structure of LM-1. Further verification for this was obtained by the observation of strong nOe's between these two signals (15%, 17%), suggesting these hydrogens are in close proximity, and by the fact that no cross peaks were observed between these two signals in the COSY spectra of LM-1.

More proof of the assignment of the hydrogen signals at δ 2.85 and δ 3.63, and the stereochemistry at C_{12} and C_{13} was obtained by the nOe and decoupling data obtained on these signals. NOe's are observed between these two signals (6.4%, 8.3%), and coupling observed between these signals is small (1.5 Hz). No nOe's would be expected and the coupling would be greater between these two signals if one or the other centers were of opposite stereochemistry.

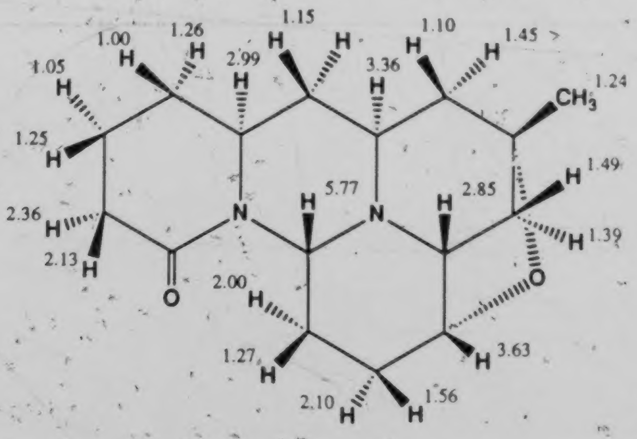
Further assignments of the ^{1}H NMR spectrum of LM-1 can be made from the following. The hydrogen signal at δ 5.77 (dd, J = 11, 5.0 Hz) shows a cross peak in the ^{1}H-^{1}H COSY spectum only to signals at δ 2.00 and δ 1.27 (Figure 12). These signals can thus be assigned to the hydrogens on C_{10}. Decoupling of the signal at δ 5.77 confirms a 5.0 Hz coupling between this signal and δ 1.27, and an 11 Hz coupling between this signal and the hydrogen signal at δ 2.00. The large coupling between δ 5.77 and δ 2.00 suggests that the hydrogen atoms giving rise to these signals are antiperiplanar.

Figure 10: Some ^{1}H NMR nOe's observed for LM-1.



The signal at δ 2.00 is important in establishing the relative assignments of the signals at δ 3.36 and δ 2.99 corresponding to hydrogens at C_5 and C_7. A strong nOe is observed from δ 3.36 to δ 2.00 (10%), and a weaker one from δ 2.99 to δ 2.00 (6.5%). A molecular model of the proposed structure of LM-1 suggests that the axial hydrogen atom at C_{10} (δ 2.00) is closer to the hydrogen at C_7 than to C_5, hence the signal at δ 3.36 was assigned to the hydrogen at C_7, and the signal at δ 2.99 was assigned to the hydrogen at C_5. The relative assignment of these signals is consistant with the ^1H-^1H COSY spectrum of LM-1 which shows that the signal at δ 3.36 is near one end of a spin system, whereas the signal at δ 2.99 is in the middle of the same system. The stereochemistry of the hydrogens at C5 and C7 is confirmed by nOe's which are observed between these two signals (4.7%, 6.9%). If one or the other hydrogen atoms were of opposite stereochemistry, nOe's would not be observed between these signals, and if both hydrogen atoms were of opposite stereochemistry, nOe's would not be observed between these signals and the signal at δ 2.00. Thus, the nOe's observed between δ 3.36, δ 2.99, and δ 2.00 are further proof for the proposed structure and stereochemistry of LM-1. Other ^1H NMR assignments for LM-1 (Fig. 11) are based on the ^1H-^1H COSY, decoupling, and nOe data obtained on this compound.

Figure 11: ^1H NMR assignment for LM-1 (δ in ppm, solvent - benzene d6).



Lycopodium meridionale

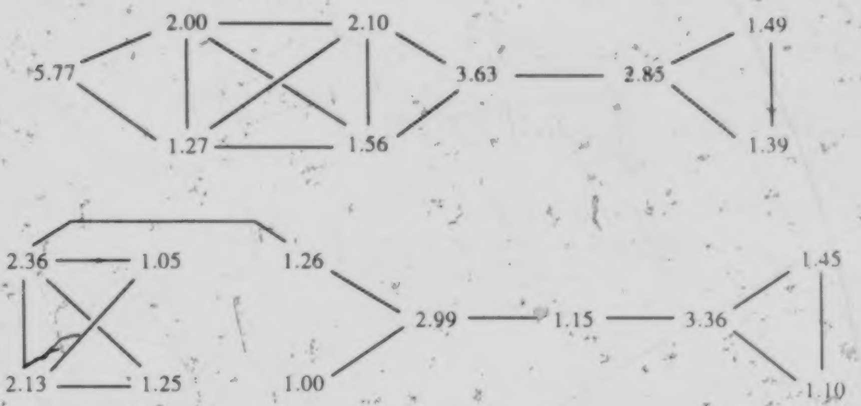
Table17: ^{1}H NMR nOe data (benzene d_6) for LM-1.

Signal Irradiated (δ):		**nOe (%)**	
H_9	5.77	H_{13}	2.85 (15)
H_{12}	3.63	H_{13}	2.85 (6.4)
		H_{11a}	1.56 (5.8)
		H_{14e}	1.39 (3.5)
H_7	3.36	H_5	2.99 (6.9)
		H_{10a}	2.00 (10)
		H_{8e}	1.45 (10)
H_5	2.99	H_7	3.36 (4.7)
		H_{10a}	2.00 (6.5)
		H_{3a}	1.05 (10)
H_{13}	2.85	H_9	5.77 (17)
		H_{12}	3.63 (8.3)
		H_{14e}	1.39 (3.9)

Table 18: ^{1}H NMR Decoupling data (benzene d_6) for LM-1.

Signal Irradiated (δ):		**Observed change at (δ):**		**Coupling (Hz)**
H_9	5.77	H_{10a}	2.00	11.0
		H_{10e}	1.27	5.0
H_{12}	3.63	H_{13}	2.85	1.5
		H_{11a}	1.56	5.0
H_7	3.36	H_{8e}	1.45	5.5
		$H_{6?}$	1.15	11.0
		H_{8a}	1.10	11.0
H_5	2.99	H_{4e}	1.26	3.3
		$H_{6?}$	1.15	8.2
		H_{4a}	1.00	10.0
H_{13}	2.85	H_{12}	3.63	1.5
		H_{14e}	1.39	2.5
H_{2e}	2.36	H_{2a}	2.13	17.2
		H_{3a}	1.05	2.5

Figure 12: ^{1}H NMR COSY Spin Systems for LM-1
(Chemical shifts in δ, solvent = d6-benzene).



To further establish the structure of LM-1 it was subject to a number of chemical transformations. Reaction of LM-1 with boron triflouride-diethyl etherate in acetic anhydride resulted in cleavage of the ether ring. Analysis of the product by ^{1}H NMR, ^{13}C NMR and GC/IR showed the presence of two isomeric compounds both containing a double bond and an acetate unit. The ^{1}H NMR spectrum of the mixture displays a signal at δ 1.96 ppm (3H, s) corresponding to an acetate unit, and a signal at δ 5.22 ppm (1H, s) corresponding to an allylic hydrogen of the major component, and a signal at δ 1.95 ppm (3H, s) and δ 5.17 ppm (1H, s) for the acetate unit and the allylic hydrogen of the minor component (Figure 14). The ^{13}C NMR spectrum of the mixture shows signals at δ 132.9 ppm and δ 119.2 ppm corresponding to the allylic carbon atoms of the major component and at δ 130.9 ppm and δ 121.7 ppm for the minor one. As well, a signal in the ^{13}C NMR spectrum of the mixture is observed at δ 22.8 ppm for the acetate unit of the major component, and one at δ 22.7 ppm for the minor component. In the GC/IR spectrum of the mixture, the presence of two compounds is established by the GC tracings. The gas phase infrared spectra of each of these compounds show very intense absorptions around 1755 cm^{-1} and 1229 cm^{-1}, indicating the presence of acetate units.

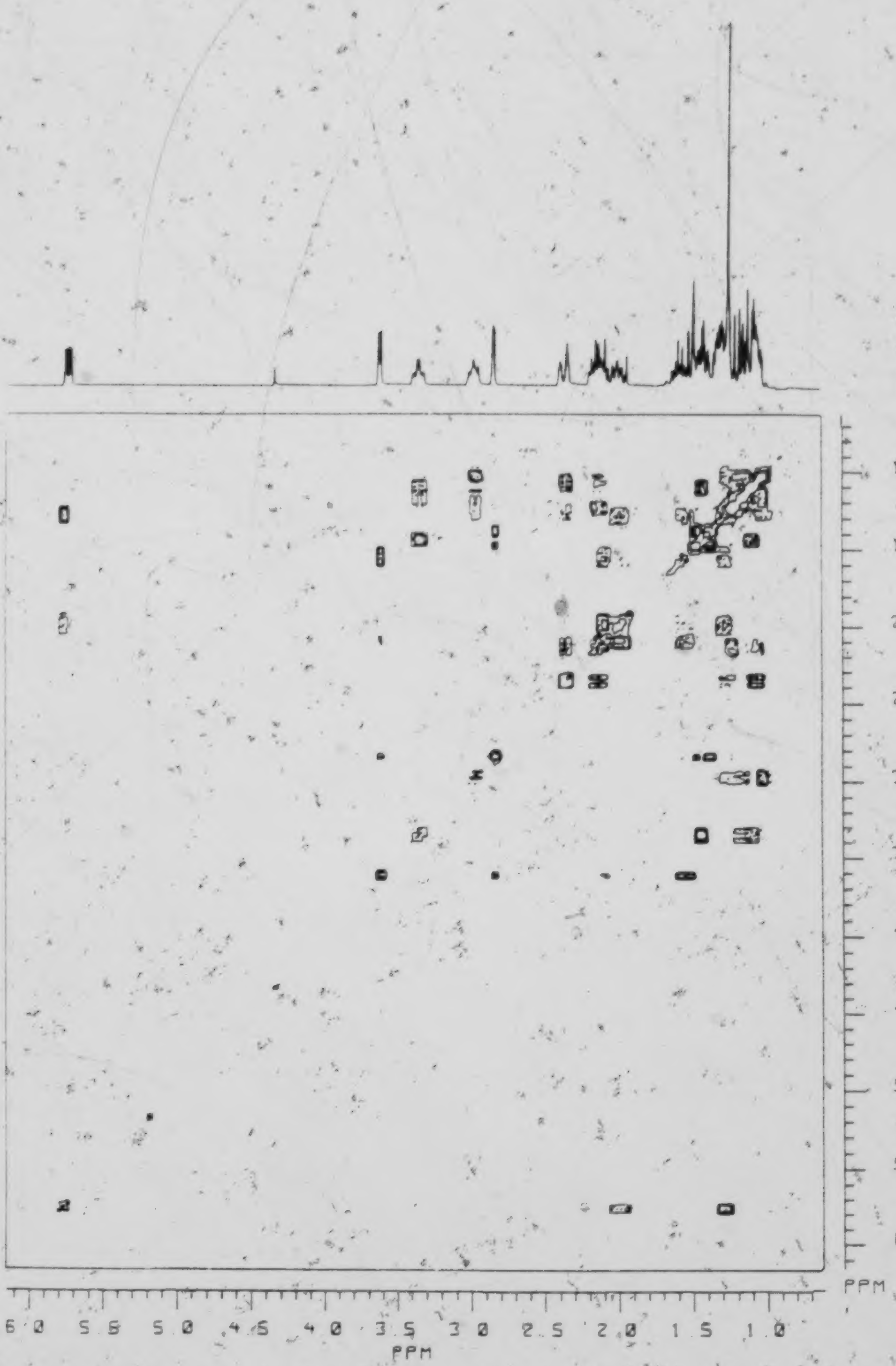
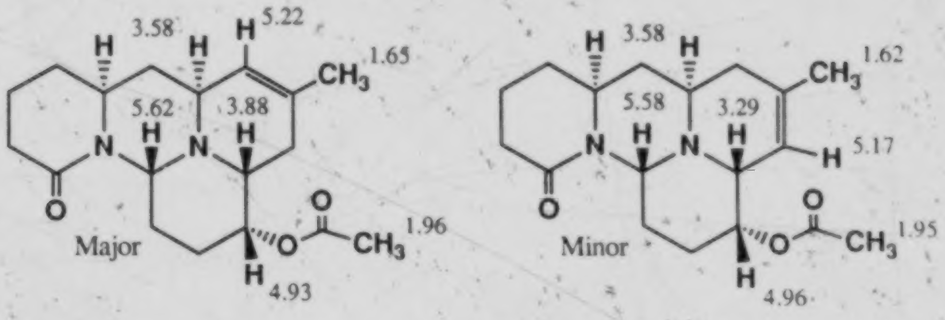


Figure 13: ^1H-^1H NMR COSY spectrum (benzene-d_6) of LM-1.

Figure 14: ^{1}H NMR assignment of LM-1-A (CDCl$_3$,δ in ppm).



Separation of the two compounds was not successful, and so the mixture was subject to hydrogenation in 95% ethanol using 10% palladium on carbon for three days. After filtration of the catalyst and evaporation of the solvent, one major product (LM-1-A-H) was obtained after chromatography of the product over alumina. The ^{1}H NMR shows a doublet (J = 6.5 Hz) at δ 0.87 corresponding to a methyl group, indicating hydrogen addition to C$_{15}$. Furthermore, its mass spectrum reveals a molecular ion (m/z 320) with an additional 2 mass units compared to LM-1-A, indicating the addition of two hydrogens, and it is very similar to the mass spectrum for O-acetyllycocernuine. LM-1-A-H is not identical with O-acetyllycocernuine however since other spectral data (^{1}H NMR, ^{13}C NMR, IR, GC/IR,) is not identical with that of O-acetyllycocerniune (see experimental section and Table 23). In spite of this, the stereochemistry at C$_9$, C$_{12}$, and C$_{13}$ is the same as lycocerniune and LM-1 by the following data. The ^{1}H NMR shows a deshielded hydrogen at δ 5.42 ppm (dd, 11.5 Hz, 2.5 Hz) which has been assigned to C$_9$, and the coupling constants are similar to those for lycocernuine. A strong nOe (11.7 %) is observed between this hydrogen and a hydrogen at δ 3.28 ppm, assigned to C$_{13}$. This hydrogen signal shows an nOe back to δ 5.42 ppm (14.3%) and to δ 4.68 (10.2 %) assigned to C$_{12}$. This information indicates that these three hydrogens are in close proximity to each other. As in lycocernuine and LM-1, the coupling between the hydrogens at C_{12} (δ 4.68) and C_{13} (δ 3.28) is very small (>2 Hz), indicating a similar conformation at these positions for this compound.

Since hydrogenation of the double bond would appear to favor the top face of the molecule from examination of a molecular model of this compound, the methyl group at C_{15} on LM-1-A-H presumably has the opposite stereochemistry compared to O-acetyllycocernuine. This appears to be the only difference between the two compounds. The hydrogenation of O-acetylcarolinianine leads to this same compound (15-epi-O-acetyllycocernuine) [33]. Hence the minor reaction product in the mixture of LM-1-A is O-acetylcarolinianine (see Figure 16), and the structure of LM-1 is further verified.

Figure 15: ^{1}H NMR spectral assignment for LM-1-A-H ($CDCl_3$, δ in ppm).

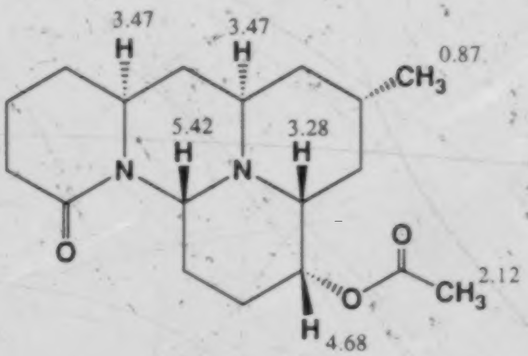
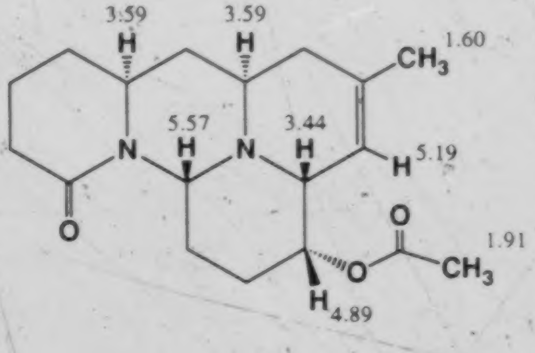
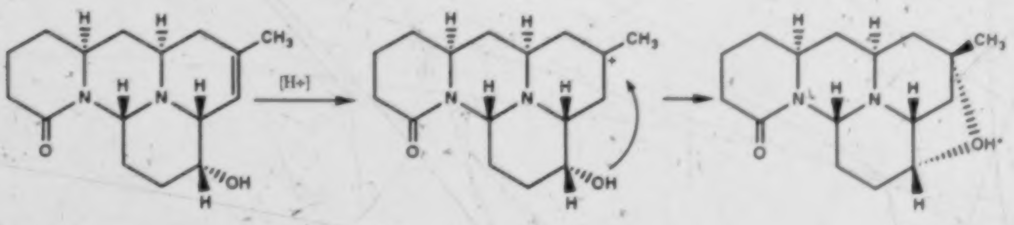


Figure 16: ^{1}H NMR spectral assignment for O-acetylcarolinianine ($CDCl_3$, δ in ppm) [33].



One other reaction was performed to establish the structure of LM-1. The mixture of ring cleaved products of LM-1 (LM-1-A) was heated in 5% hydrochloric acid on a steam bath, and the products were analyzed by GC/IR. After heating for 17 hours two new products were observed in the mixture along with the starting material. They appear to be the hydrolysis products of LM-1-A by IR analysis since both show no absorption in the region of 1755 cm^{-1} and 1229 cm^{-1} in their infrared spectra, and both show a close but not identical match in the GC/IR library to lycocernuine. After a further 67 hours of heating on a steam bath, the starting material was no longer observed by GC/IR. The hydrolysis products were observed as the major products along with one other compound in minor amounts. The infrared spectrum of the minor product shows a very close match in the GC/IR library to LM-1.

Figure 17: Acid catalyzed formation of LM-1.

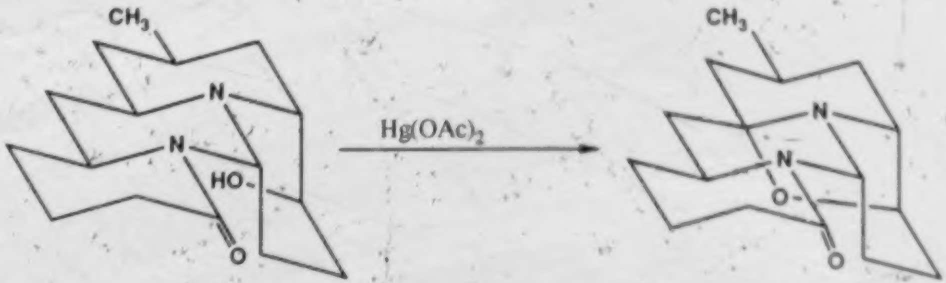


The reaction does not proceed to a very great extent. Even after 67 hours, the minor amount of LM-1 produced is not detectable by ^{1}H NMR analysis of the final product mixture. The low yield may be accounted for by the 'poor competition' between the basic nitrogen atom and the double bond for the proton. Because of the undesirable effect of having two positive charges in such close proximity, only a very small amount of material would be protonated at the double bond if the nitrogen were already protonated. The low yield supports the claim that LM-1 is a natural product and not an artifact, since LM-1 accounts for a substantial fraction of the total alkaloid content of the plant material.

Lycopodium meridionale

A number of reactions were performed in an attempt to transform lycocernuine into LM-1. Lycocernuine is oxidized to an ether when treated with mercuric acetate under the appropriate conditions (Figure 18) [36b]. Also, dihydrolycopodine can be oxidized to an ether using lead tetraacetate (see page 21). From these observations, it was thought that lycocernuine might be transformed to LM-1 when treated with lead tetraacetate under the appropriate conditions. A number of attempts were made using different solvents, and using either heat or light to initiate the reaction, but all attempts failed or gave the same product as the reaction using mercuric acetate. Analysis of the reaction products by ^{1}H NMR always showed the presence of a doublet around δ 0.95, corresponding to a methyl group, whereas the methyl group in LM-1 appears as a singlet around δ 1.34 ppm.

Figure 18: Oxidation of lycocernuine with mercuric acetate.



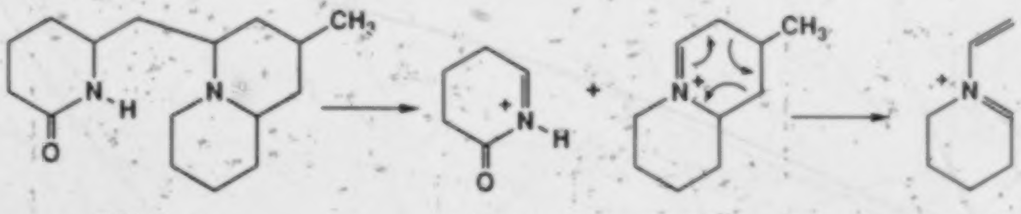
Identification of LM-3.

The last compound examined in this study from the alkaloid extracts of *Lycopodium meridionale*, LM-3, was isolated as a colorless oil that solidified upon standing, and melted at 81-82°C. The molecular formula was determined from the high resolution mass spectrum as $C_{16}H_{28}N_2O$ and confirmed by a chemical ionization (NH_3) mass spectrum which shows an ion at *m/z* 265 (100%) corresponding to M+H. The presence of a secondary amide functionality was established by a solid phase ($CHCl_3$ cast) infrared spectrum which shows a broad band at 3215cm^{-1} and 3018 cm^{-1} and a strong band at 1664 cm^{-1}, and a solution infrared spectrum which shows a sharp single peak at 3400 cm^{-1} and a strong band at 1646 cm^{-1}. The ^{1}H NMR spectrum supports this as a broad signal at δ 6.65 is observed that is exchangeable with D_2O. The presence of an amide was confirmed by a signal at δ 171.5 in the ^{13}C spectrum of LM-3.

From the molecular formula, $C_{16}H_{28}N_2O$, there are four sites of unsaturation in the molecule, and the amide carbonyl group accounts for one site of unsaturation. Since there is no spectral evidence for double or triple bonds in LM-3 the three remaining sites can be accounted for if LM-3 is tricyclic.

The mass spectra of LM-3 is particularly informative in the structure determination of this compound. The molecular ion established a molecular formula of $C_{16}H_{28}N_2O$ for LM-3. The base peak is observed at *m/z* 152, and corresponds to an ion with a formula $C_{10}H_{18}N$. Other ions are observed at *m/z* 110 ($C_7H_{12}N$, 9.5%), and *m/z* 98 (C_5H_8NO, 1.2%), and are suggestive of the following structure and fragmentation.

Scheme 3: Important mass fragmentation modes for LM-3 (HREIMS).



LM-3 *m/z* 98 *m/z* 152 *m/z* 110

The ^{1}H NMR and ^{13}C NMR spectra are in agreement with the proposed structure. The ^{1}H NMR spectrum displays in addition to the broad signal at δ 6.65 ppm, five downfield signals (δ 3.46, 3.15, 3.04, 2.92, 2.61 ppm, $CDCl_3$) which can be assigned to hydrogens on carbons next to the nitrogen atoms, and a doublet (6 Hz) at δ 0.88 ppm reveals a methyl group attached to a tertiary carbon atom. As well as the signal at δ 171.5 ppm assigned to the carbonyl carbon, the ^{13}C NMR spectrum shows three additional downfield signals (δ 54.7 (CH), 2 x 51.2 (CH), 49.5 (CH_2) ppm, $CDCl_3$) which can be assigned to the four other carbon atoms adjacent to nitrogen. Two of these signals are unusually broad (δ 54.7, δ 51.2). When the ^{13}C spectrum of LM-3 was acquired at 55°C, these peaks became sharper and an additional peak was observed under the signal at δ 51.2 (Figure 19). The chemical shifts of the other ^{13}C NMR signals are consistent with the remaining eleven carbon atoms in LM-3.

A partial ^1H NMR assignment for LM-3 (Figure 20) has been made from the ^1H NMR nOe (Table 19) and decoupling (Table 20) data obtained on this compound. The downfield hydrogens were assigned as follows. Strong irradiation of the broad D_2O exchangable signal at δ 6.65 ppm (assigned to the amide hydrogen) shows a sharpening of the signal at 3.46 ppm. Hence, this signal was assigned to the hydrogen at C_5. An nOe (1.3%) was observed between this signal and δ 2.92 ppm (1.3%, 2.7%), suggesting that the two hydrogen atoms corresponding to these signals are in close proximity, hence the signal at δ 2.92 ppm was assigned to the hydrogen at C_7. Decoupling of the signal at δ 3.15 ppm reveals that this signal is coupled to the signal at δ 2.61 ppm by 12.0 Hz, and suggests that the hydrogens corresponding to these signals are on the same carbon atom. Therefore, these signals are assigned to the hydrogens on C_9. The remaining signal at δ 3.04 ppm was assigned to the hydrogen on C_{13}. An nOe effect is observed between this signal and the one at δ 2.61 ppm (5.6%, 5.3%). If the stereochemistry is correct at C_{13}, and the other assignments are correct, this nOe implies that the hydrogens at δ 2.61 ppm (C_9) and δ 3.04 ppm (C_{13}) are in close proximity and may be in axial positions as shown in Figure 20. Other assignments are based on these assignments and on ^1H decoupling and ^1H NMR nOe studies.

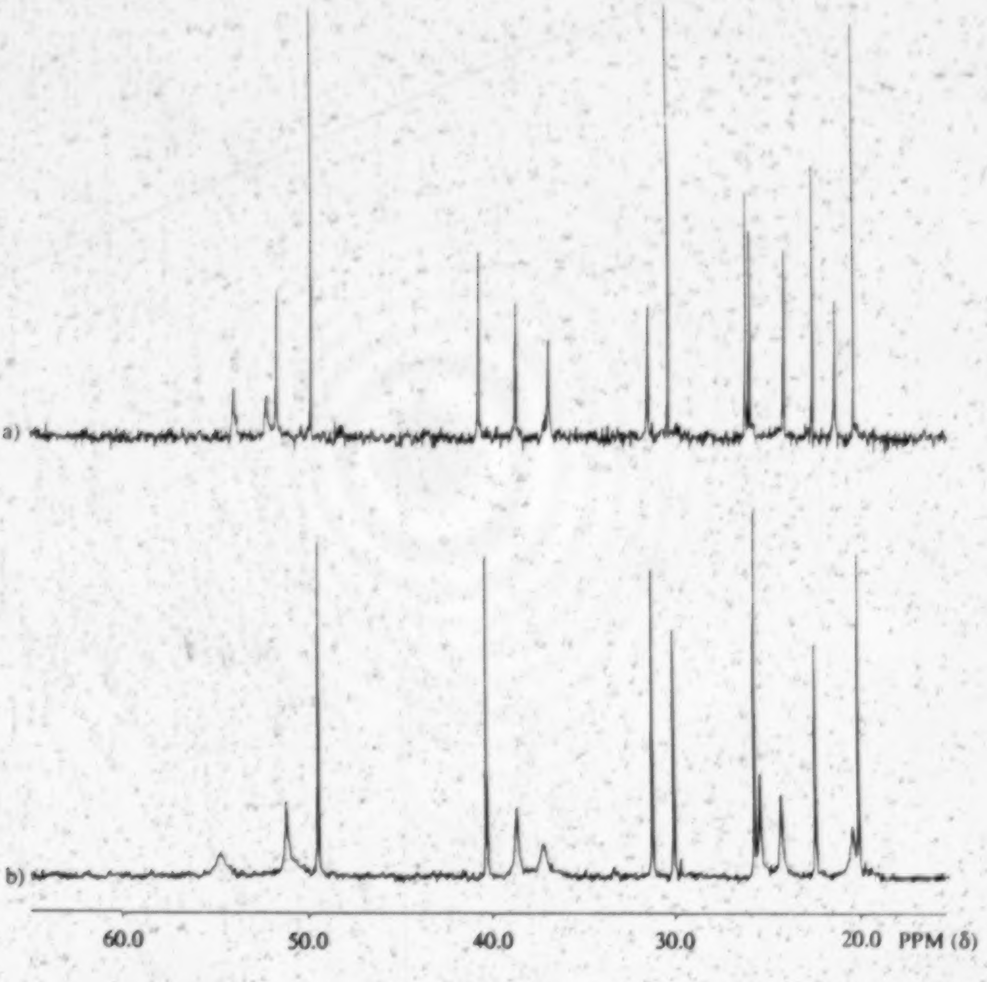


Figure 19: Partial ^{13}C NMR spectra ($CDCl_3$) of LM-3 at: a) 55°C and b) 25°C.

Figure 20: Partial ^1H NMR assignment of LM-3 ($CDCl_3$).

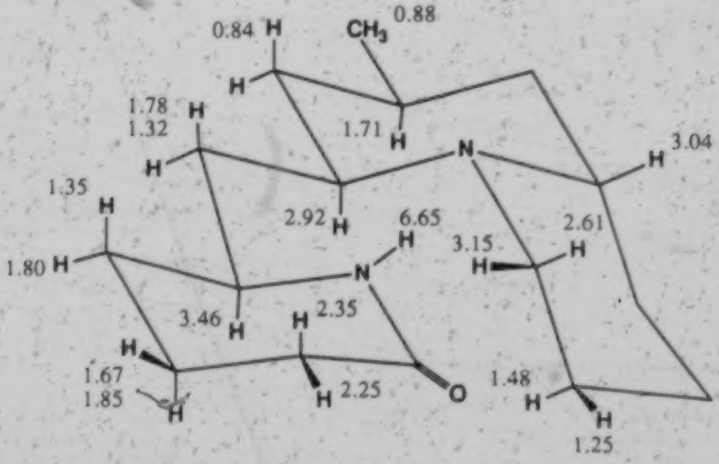


Table 19: ^1H NMR nOe data ($CDCl_3$) for LM-3.

Signal Irradiated (δ):		**nOe (%)**	
H_5	3.46	H_7	2.92 (1.3)
		H_{4e}	1.80 (3.1)
		$H_{6'e'}$	1.32 (4.5)
H_{9e}	3.15	H_{9a}	2.61 (25.7)
		$H_{6'a'}$	1.78 (7.4)
H_{13}	3.04	H_{9a}	2.61 (5.6)
		H_{10a}	1.48 (4.3)
		$H_?$	1.21 (11.2)
H_7	2.92	H_5	3.46 (2.7)
		H_{15}	1.71 (16.4)
		H_{10a}	1.48 (4.5)
		$H_{6'e'}$	1.32 (3.2)
H_{9a}	2.61	H_{9e}	3.15 (16.2)
		H_{13}	3.04 (5.3)
		H_{10a}	1.48 (2.0)
		H_{10e}	1.25 (3.2)

Table 20. ^1H NMR Decoupling data ($CDCl_3$) for LM-3.

Signal Irradiated (δ):		**Observed change at (δ):**		**Coupling (Hz)**
N-H	6.65	H_5	3.46	>1
H_5	3.46	H_{4e}	1.80	3.5
		$H_{6'a'}$	1.78	9.0
		H_{4a}	1.35	9.5
		$H_{6'e'}$	1.32	3.5
H_{9e}	3.15	H_{9a}	2.61	12.0
		H_{10a}	1.48	3.0
		H_{10e}	1.25	3.0
H_{13}	3.04	H?	1.70	?
		H?	1.60	?
H_7	2.92	$H_{6'a'}$	1.78	4.5
		$H_{6'e'}$	1.32	7.5
		$H_{8?}$	0.84	12.0
H_{9a}	2.61	H_{9e}	3.15	12.0
		H_{10a}	1.48	10.0

A compound apparently similar to LM-3 has been isolated from *L. cernuum*, however this compound, "Base G" was never fully characterized because of the small sample size [81]. The structure of Base G was determined mainly from the following mass and infrared spectral data. MS: *m/z* (relative intensity), 264 (2), 185 (13), 153 (18), 152 (100), 150 (10), 111 (14), 110 (23), 81 (72); IR (CH_4 sol.): 3590, 3520, 3440, 3400, 1750, 1680, 1660, 1460 cm^{-1}. The infrared spectrum shows absorptions similar to LM-3 (3400, 1660 cm^{-1}) corresponding to an amide functionality, as well as additional peaks which may or may not be due to impurities in the sample of Base G. As well, the mass fragmentation peaks at 152 and 110 suggest the same sort of fragmention modes for Base G as for LM-3 (Scheme 3). It is possible that Base G and LM-3 are the same compound although this cannot be verified unless LM-3 is isolated from *L. cernuum* in the future since a sample of Base G is no longer available.

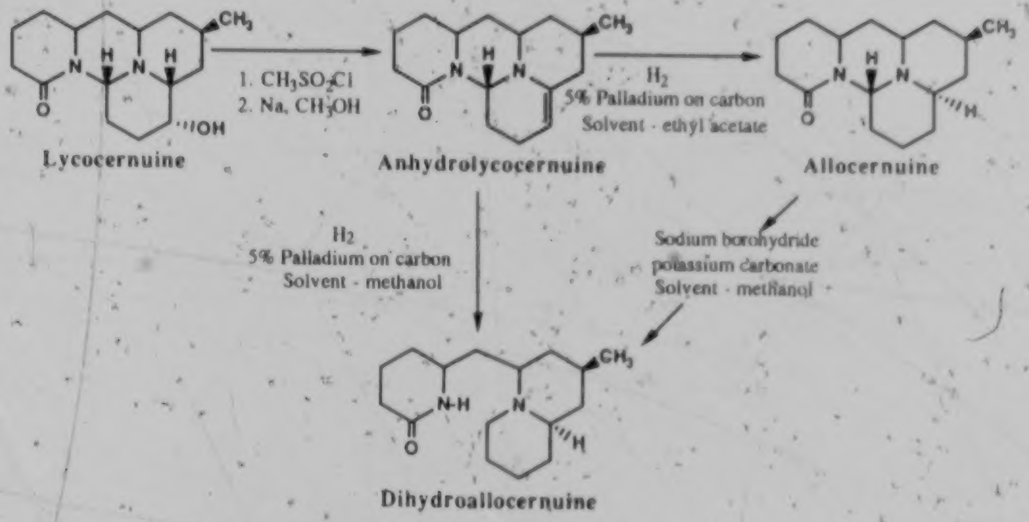
Lycopodium meridionale

Another compound apparently similar to LM-3 is dihydroallocernuine, a synthetic compound obtained from the dehydration and subsequent hydrogenation of lycocernuine [36a]. The following spectral data has been reported for dihydroallocernuine: MS: *m/z* (relative intensity), 264 (3), 166(4), 153(19), 152(100), 110(16); IR ($CHCl_3$ sol.): 3400, 1645 cm^{-1}; ^{1}H NMR: 6.30 ppm (1H, broad, amide NH), 3.3-3.7, (3-4H, complex multiplet), 0.93 (3H, doublet, 6.0 Hz). This spectral data compares well with LM-3. An authentic sample of dihydroallocernuine was no longer available for direct comparison, so attempts were made to repeat the reported chemical transformation of lycocernuine to dihydroallocerniune.

To obtain dihydroallocernuine, lycocernuine is first dehydrated to form anhyrolycocernuine. Dihydroallocernuine can be obtained from anhydrolycocernuine in one of two ways (Scheme 4). Hydrogenation of anhydrolycocernuine in methanol directly produces dihydroallocernuine. Hydrogenation of anhydrolycocernuine in ethyl acetate under the same conditions produces allocernuine which can be reduced with sodium borohydride in methanol to form dihydroallocernuine.

Early attempts at obtaining dihydroallocernuine from the hydrogenation of anhydrolycocernuine appeared to fail since the ^{1}H and ^{13}C NMR spectra of the compound obtained compared poorly with the spectra of LM-3 (although the mass spectra compared quite well). When the same compound was obtained from the sodium borohydride reduction of allocernuine, it was concluded that early attempts did not fail, and that the compound obtained is the same as that reported previously [36a].

Scheme 4: The synthesis of dihydroallocernuine.



Figures 21b and 22b show the ^1H NMR spectra of LM-1 and dihydroallocernuine. Examination of the two spectra reveals the conspicuous absence of three hydrogen signals in the downfield region of the spectrum of dihydroallocernuine compared to LM-1. Figures 19b and 23b show the ^{13}C NMR spectrums of these two compounds, and again, examination of the two spectra reveals the apparent absence of at least two carbon signals in the downfield region of the spectrum of dihydroallocernuine compared to LM-3. These observations led to the conclusion that these two compounds are structurally different.

Even with these differences it is difficult to ignore the similarities of the spectral data of these two compounds. The ^{13}C NMR spectra of both LM-3 and dihydroallocernuine show some unusually broad peaks not observed for any of the other *Lycopodium* alkaloids so far examined. This, and the fact that the mass spectra are very similar suggests there is some similarity between LM-3 and dihydroallocernuine.

The broad peaks in the ^{13}C NMR spectra suggests some conformational changes are taking place at a rate similar to the NMR time scale. The spectra were re-acquired at

55°C, and some significant changes took place (Figure 19a, 23a). Many of the broad peaks sharpen up, and the 'missing' signals appear from under a number of broad signals. Also, all of the carbon atoms of LM-3 and dihydroallocernuine can be seen in the ^{13}C NMR spectra obtained at 55°C (Table 21, Figure 24).

The ^{1}H NMR spectra of LM-3 and dihydroallocernuine were also re-acquired at 55 °C (Figures 21a, 22a). Many signals in both spectra are much sharper, and more signals are resolved. More distinct couplings can be observed for many of the signals, and the amide peak in both spectra becomes much sharper. However, the three 'missing' signals in the spectrum of dihydroallocernuine are still not unambiguously observed, although new multiplets start to appear around δ 2.00.

Table 21: ^{13}C NMR spectra of LM-3 and dihydroallocernuine at 25°C and 55°C.

	LM-3		**Dihydroallocernuine**	
Carbon number	25°C	55°C	25°C	55°C
1	171.5	172.0	172.0	171.9
2	40.4	40.6	40.8	40.7
3	31.3	31.3	31.3	31.3
4	30.1	30.2	30.6	30.6
5	*51.2	*52.1	*51.1	51.1
6	20.1	20.1	19.9	19.8
7	*54.7	*53.9	*60.2	60.3
8	*37.1	*36.7	*40.8	*40.3
9	49.5	49.7	*51.1	*50.3
10	25.5	25.7	29.8	29.8
11	24.2	23.9	*24.0	24.0
12	20.6	21.1	*33.6	*33.5
13	*51.2	51.6	*62.6	*62.6
14	*38.9	38.6	*40.8	*40.3
15	25.7	26.0	26.3	26.2
16	22.4	22.4	22.0	22.0

* Broad Peaks

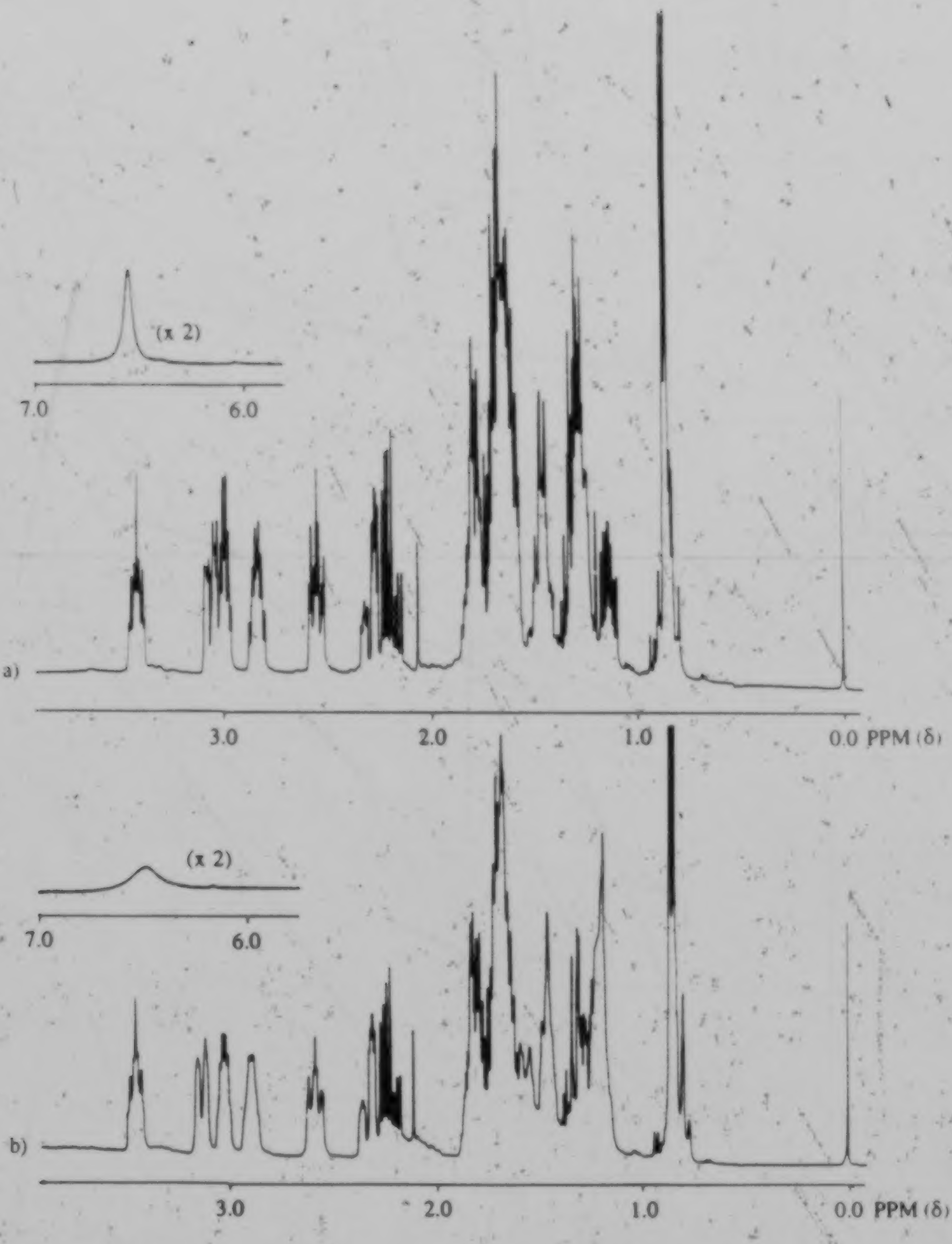


Figure 21: ^{1}H NMR spectra of LM-3 at: a) 55°C and b) 25°C.

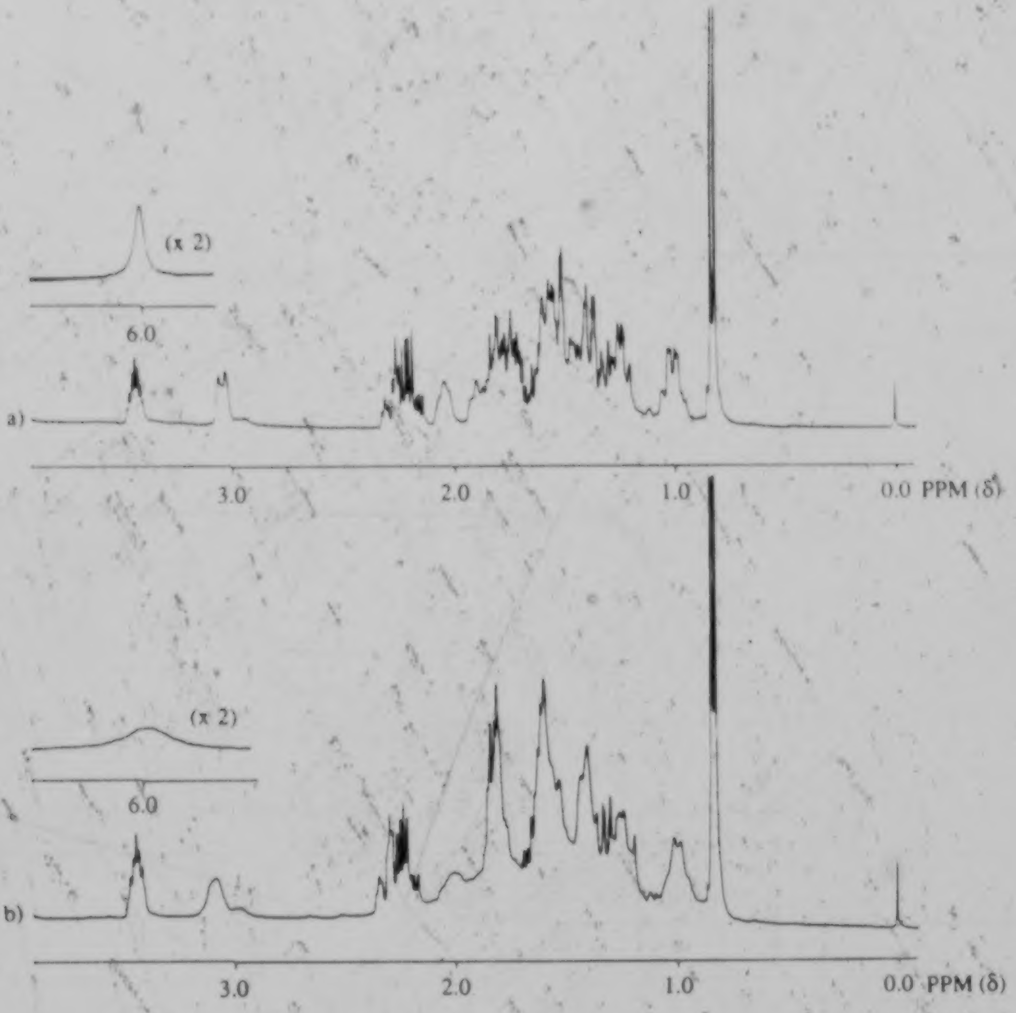


Figure 22: ^{1}H NMR spectra of dihyroallocernuine at: a) 55°C and b) 25°C.

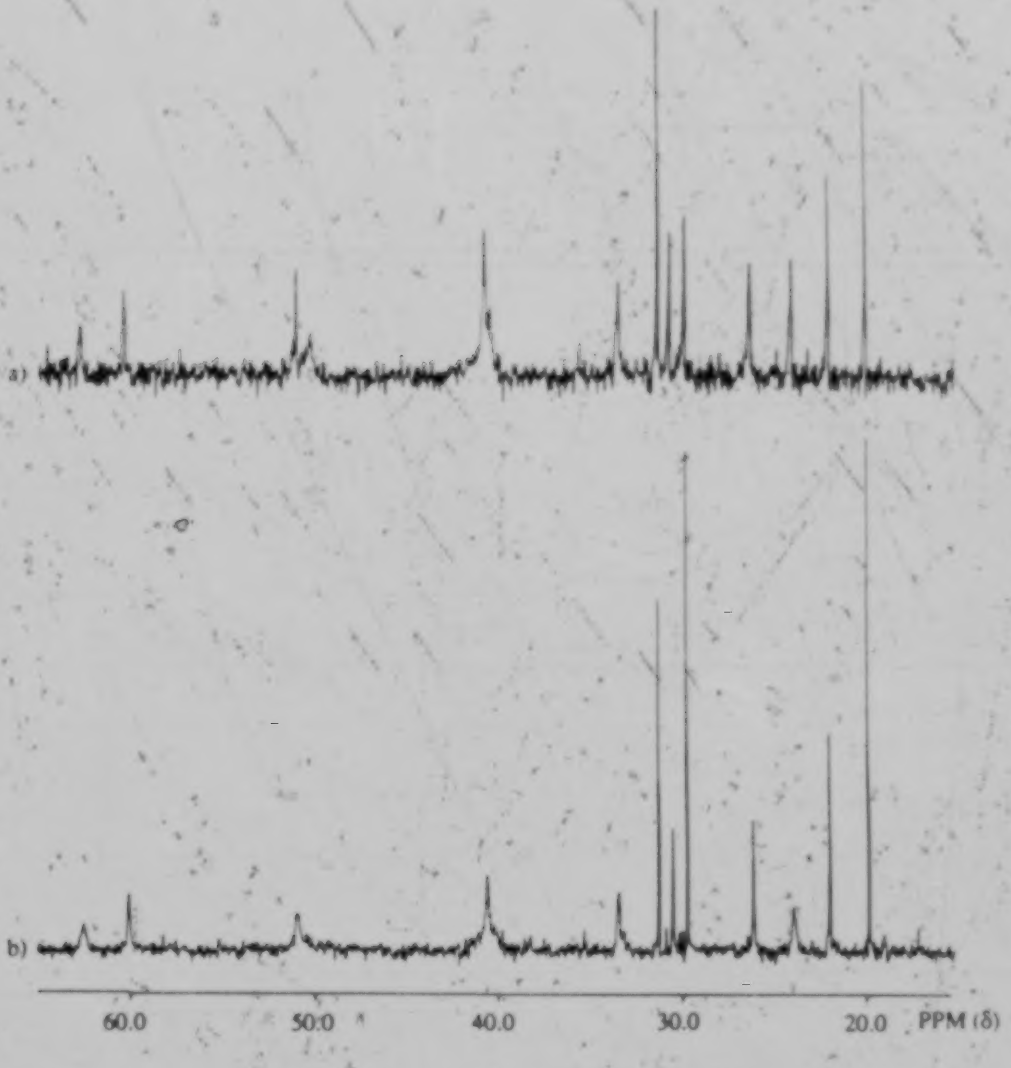


Figure 23: Partial ^{13}C NMR spectra of dihyroallocernuine at; a) 55°C and b) 25°C.

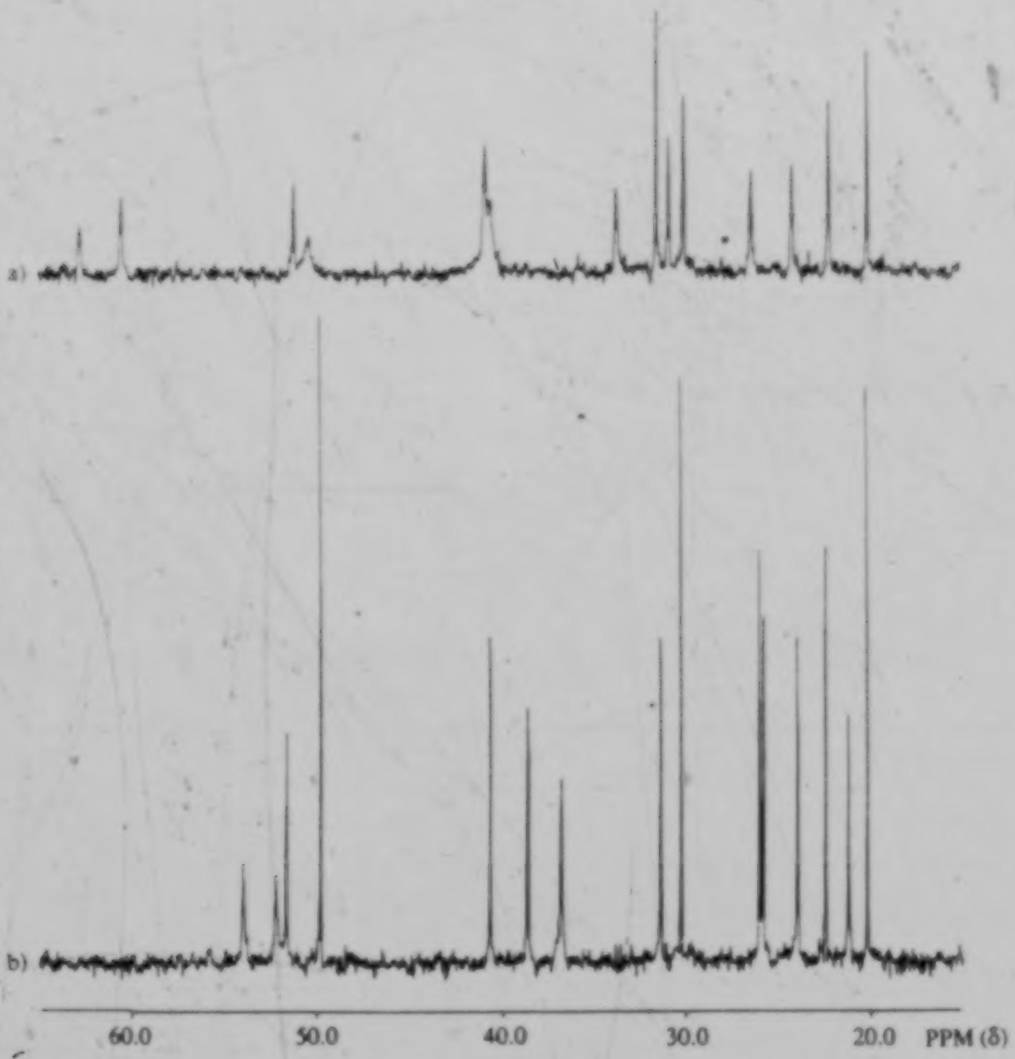


Figure 24: Partial ^{13}C NMR specta (CDCl$_3$, 55°C) of: a) dihyroallocernuine and b) LM-3.

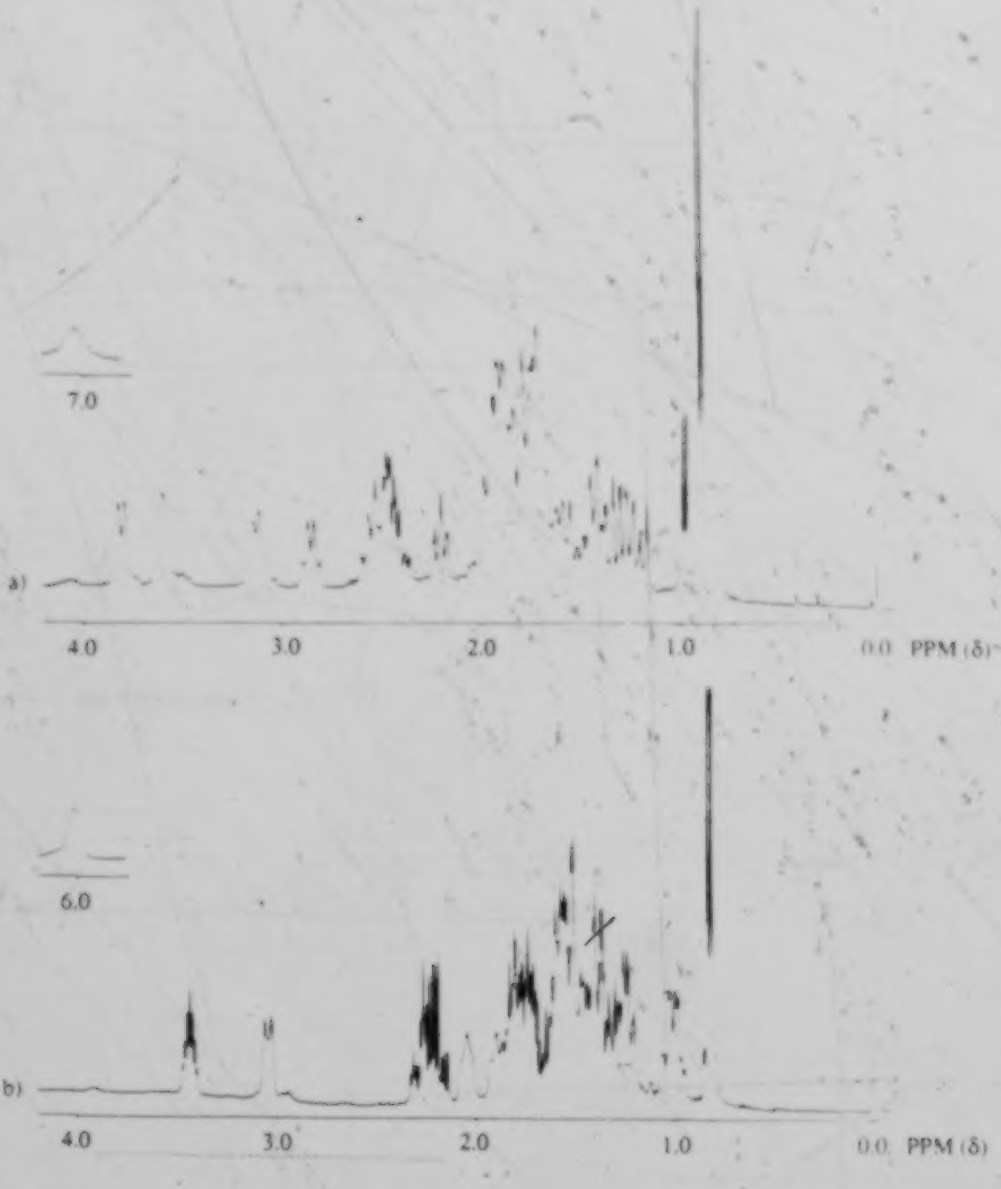


Figure 25: ¹H NMR specta of: a) dihyroallocernuine with addition of trifloroacetic acid at 25°C and b) dihyroallocernuine at 55°C.

To observe the 'missing' signals, trifloroacetic acid (TFA) was added to an NMR tube containing dihydroallocernuine, and the ^{1}H NMR spectrum was re-acquired (Figure 25). Protonation of the basic nitrogen atom by TFA should cause a downfield shift of hydrogens on attached carbons. The observed spectrum of dihydroallocernuine shows three additional signals in the downfield region, confirming the presence of these three 'missing' hydrogens. An ^{1}H NMR COSY spectrum (see Figure 26) was obtained to help establish the assignment of the ^{1}H NMR spectrum of dihydroallocernuine (Figure 27).

Figure 26: ^{1}H NMR COSY spin systems for dihydroallocernuine ($CDCl_3$+TFA) (δ in ppm).

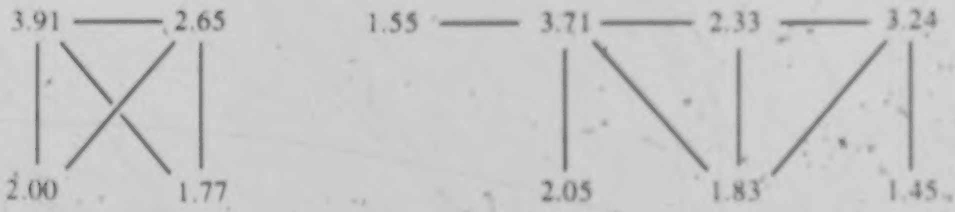
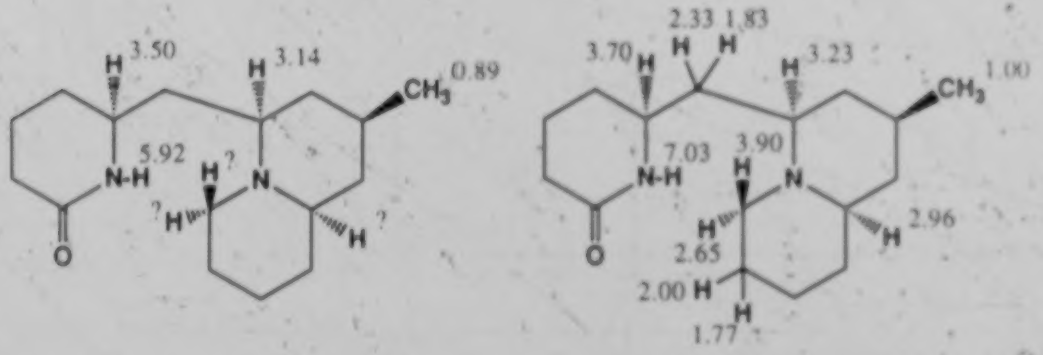


Figure 27: Dihydroallocernuine partial ^{1}H NMR assignments (δ in ppm).



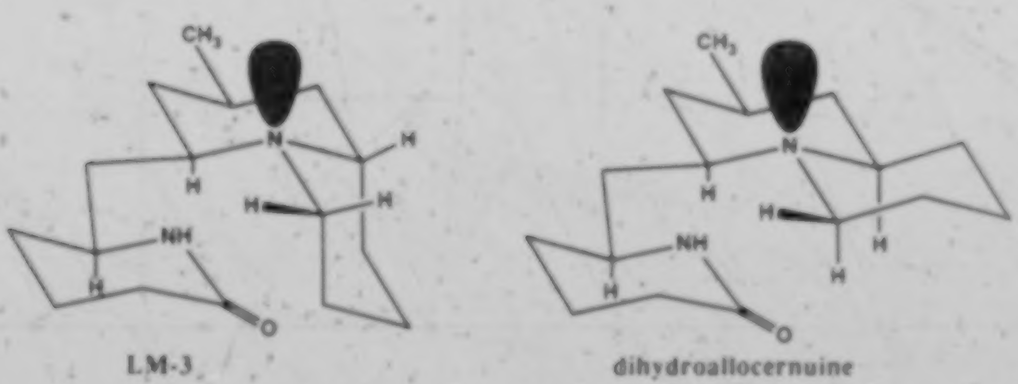
^{1}H NMR ($CDCl_3$)

^{1}H NMR ($CDCl_3$ + TFA)

The structures of LM-1 and dihydroallocernuine presumably differ in stereochemistry at C_{13}. The stereochemistry of LM-3 at C_{13} is most likely the same as other naturally occurring alkaloids of this type. Dihydroallocernuine is obtained from the hydrogenation of allocernuine. Hydrogenation presumably takes place from the less hindered bottom face of the molecule, leading to a compound with the opposite stereochemistry at C_{13}.

Examination of the structures of LM-1 dihydroallocernuine indicate that a change in the stereochemistry at C_{13} can change the overall configuration of the molecule (Figure 28). Hence, although the mass spectra of these compounds suggest that the carbon skeletons are the same, LM-3 and dihydroallocernuine are conformationally different enough to have substantially different NMR spectra.

Figure 28: Configurations for the proposed structures of LM-3 and dihydroallocernuine



The broadness of the ^{13}C NMR signals and the sharpening of these signals as well as ^{1}H NMR signals at 55°C for both compounds can be explained by the presence of conformational isomers in solution. There is a relatively small barrier to inversion of the stereochemistry at the basic nitrogen atom for both compounds. The resulting interconversion would place hydrogen and carbon atoms around this center in different electronic environments depending on the conformation, and hence only the average signal for these atoms would be observed in the NMR spectra. As well, LM-3 and the N-inverted form of dihydroallocernuine are "cis-decalin" like and are conformationally mobile. There is also the problem of the slowly exchanging amide nitrogen. It is thus not surprising that the broad peaks are observed.

Assignment of ^{13}C NMR spectral data for some cernuane type alkaloids.

The ^{13}C NMR spectra of a number of alkaloids of the cernuine type were acquired during this study, none of which have so far appeared in the literature. The tentative assignment of the ^{13}C NMR signals of these compounds was made by comparing these signals with structural changes between these compounds. For example, comparison of the spectra of cernuine and lycocernuine shows that most of the signals are very similar. Assignments can be made easily for the carbonyl carbon (C_1), the carbon atom between the two nitrogen atoms (C_9), and the methyl group and adjacent carbon atom (C_{15} and C_{16}) for cernuine and lycocernuine from anticipated values [82,83] and by examination of the multiplicities of the signals. However, there are three signals in each of the spectra that can be assigned to carbon atoms adjacent to nitrogen, but the exact assignment cannot be made (Table 22).

Table 22: Comparison of some ^{13}C NMR signals for cernuine and lycocernuine (δ ppm).

Cernuine	Lycocernuine
46.1	49.0
50.2	51.0
57.5	58.6

By comparing these three signals of cernuine and lycocernuine, it is observed that one of these signals is shifted downfield by 3 ppm in lycocernuine while the other two are very similar for both compounds. Examination of the structures of cernuine and lycocernuine suggest that the expected differences would be near the area of the hydroxyl group in lycocernuine (C_{12}). Therefore, the carbon atom shifted by 3 ppm in lycocernuine (δ 49.0) is assigned to C_{13}. This kind of process was used to assign the ^{13}C NMR spectra of the compounds in the following table. However, some of these assignments must be considered tentative.

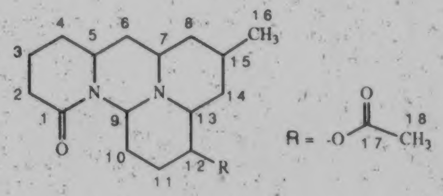
Conclusions.

The problem of the taxonomy of the *Lycopodium* species was pointed out earlier in this work including the problem with the taxonomic identification of *Lycopodium meridionale*. From the type of alkaloids identified in this work, it is apparent that this species is related closely to other species in the *Lepidotis* section which contain alkaloids of the cernuane type. Also, by comparison of the major alkaloids found in *L. meridionale* with those in the closely related species *L. carolinianum*, the structure and content of these alkaloids is different enough to suggest that these plants are not varieties of the same species, although they are closely related.

Table 23: ^{13}C NMR Assignments of some cernuane type *Lycopodium* alkaloids.

Carbon Number	Anhydro-lycocernuine	Cernuine	Allocernuine	Lycocernuine	LM-1	LM-3	*Dihydro-allocernuine	O-acetyl-lycocernuine	LM-1-A-H	LM-1-A (major)	LM-1-A (minor)
1	168.3	168.1	168.8	168.4	168.8	171.5	170.9	168.4	168.4	168.3	168.5
2	39.4	39.3	38.4	38.0	39.1	40.4	40.7	37.1	38.6	37.4	37.4
3	32.4	33.0	33.2	33.2	32.9	31.3	31.3	33.1	32.9	33.0	32.5
4	29.3	30.5	31.0	30.5	30.8	30.1	30.6	30.4	30.4	30.3	30.5
5	50.2	50.6	55.2	51.0	53.0	51.2	51.1	50.9	51.7	50.4	52.5
6	18.7	20.0	19.0	19.3	20.0	20.1	19.8	19.2	19.4	19.0	19.6
7	54.1	57.5	58.3	58.6	62.3	54.7	60.3	57.6	57.0	59.0	56.7
8	40.3	41.2	35.5	41.8	43.1	37.1	40.3	41.6	42.9	119.2	40.2
9	64.5	67.2	63.5	67.3	66.3	49.5	50.3	66.8	66.9	65.9	67.2
10	18.1	19.3	17.0	15.9	13.9	25.5	29.8	16.4	16.0	16.6	15.6
11	23.4	24.5	27.7	33.8	24.9	24.2	24.0	30.0	29.0	21.2	21.2
12	103.8	22.3	30.0	71.0	74.8	20.6	33.5	72.4	70.7	68.7	71.0
13	145.0	46.1	46.5	49.0	48.4	51.2	62.6	48.6	46.3	45.8	48.9
14	40.7	42.0	30.1	41.8	44.3	38.9	40.3	41.7	42.9	41.8	121.7
15	31.6	25.3	32.2	26.4	80.8	25.7	26.2	26.0	33.6	132.9	130.9
16	21.1	22.2	22.0	23.0	25.7	22.4	22.0	22.9	22.9	28.8	29.4
17								170.1	170.4	169.8	170.3
18								22.0	21.5	22.8	22.7

* Acquired at 55°C



EXPERIMENTAL - General

Woelm (activity III/20 mm) neutral alumina was used for column chromatography. E Merck silica gel 60 (230-400 mesh) was used for flash column chromatography. Pharmacia Sephadex LH20 was used for gel filtration chromatography. Analytical thin-layer chromatography (TLC) was carried out on alumina precoated TLC plates (E. Merck DC-Alufolien, Alumiumoxide 60 F_{254} neutral type E 0.2 mm thickness) and on silica gel precoated TLC plates (E. Merck DC-Alufolien, Kieselgel 60 F_{254} 0.2 mm thickness). The chromatograms were examined under ultraviolet light (254 or 350 nm). Visualization was completed by dipping into Dragendorff's reagent (solution A: 1.7 g of basic $Bi(NO_3)_3 \bullet 5H_2O$ in 100 ml of water:acetic acid (4:1); solution B: 40 g of KI in 100 ml of water; dipping solution: 20 ml solution A, 5 ml solution B and 70 ml water) or by dipping into a 3% phosphomolybdic acid solution (10 g of $MoO_3.H_3PO_4$, 1.25 g of $Ce(SO_4)_2$, 12 ml concentrated H_2SO_4 diluted to 250 ml with water), and charring on a hot plate (300°C). Certripical counter-current chromatography was performed on an ITO multi-layer coil separator - extractor using a Scientific Systems Inc. model 300 LC pump, and an Isco V4 variable wavelength absorbance detector.

High resolution electron impact mass spectra (HREIMS) were recorded on an AEI MS-50 mass spectrometer coupled to a DS-50 computer. Chemical ionization mass spectra (CIMS) were recorded on an AEI MS-12 mass spectrometer with ammonia as the reagent gas. Fourier transform infrared (FTIR) spectra were recorded on a Nicolet FTIR 7199 interferometer. Ultraviolet (UV) spectra were recorded on a Hewlett Packard 8450A diode array spectrophotometer. Optical rotations were recorded on a Perkin Elmer 141 polarimeter. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on Bruker WM-360 and WH-300 (operating at 75.5 MHz ^{13}C) spectrometers. Chemical shifts are reported in parts per million (δ value from tetramethylsilane (TMS)), coupling constants, J, are expressed in cycles per second (Hertz, Hz), and the following abbreviations are used: m=multiplet, s=singlet, d=doublet, t=triplet, q=quartet, br=broad.

The residual solvent was used as the internal standard, $CDCl_3$: ^1H δ 7.26 ppm; ^{13}C δ 77.0 ppm; acetone-d_6: ^1H δ 2.04 ppm, ^{13}C δ 29.0 ppm; MeOH-d_4: ^1H δ 3.30 (methyl) ppm, ^{13}C δ 49.00 ppm; benzene-d_6: ^1H δ 7.15 ppm, ^{13}C δ 128.00 ppm relative to TMS. Melting points were measured on a Zeitz-Wetzlar or a Thomas model 40 melting point apparatus and are uncorrected.

Reagent grade solvents were distilled prior to use. Skelly B or Skellysolve B refers to Skelly Oil Company light petroleum, bp 62-70°C. Analytical grade diethyl ether (ACS 288) was used without further purification.

EXPERIMENTAL - The 'L' Numbered Alkaloids

Materials and methods.

The alkaloids used in compiling the library of spectra are listed in order of increasing molecular weight in Table 11 together with the molecular formula.

GC/MS.

Alkaloid samples were prescreened by GC using the conditions described below. Initially retention indices were calculated with respect to lycopodine. Over the time period of this study, however, the retention index of some standards changed enough to make this data unacceptable for identification purposes. Analysis of the alkaloid sample by GC/MS was performed on a HP5985 MS system using an HP5840A GC with a 18835 splitless capillary inlet. The samples (1-2μL, c=1mg/mL) were injected onto a fused silica capillary column (12 m x 0.32 mm id) coated with an SE-54 equivalent (Conditions: Flow rate 4 ml/min; Carrier Gas He; Temperatures: injector 280°C, detector 300°C, oven 80°C (1min), 80-300°C at 20°/min, 300°C (5 min)). The GC was connected to the mass spectrometer by an open split interface (MS : positive ion mode; ionizing beam 70 eV; temperatures: source 200°C, transfer line 300°C; mass range 35-500; 50 scans/min). The MS of authentic samples of alkaloids were entered into a GC/MS library and test samples identified by computer match when possible [20]. Analysis of the alkaloids by GC/IR was performed on a HP5965A IRD system. The samples (1-2μL, c=1mg/mL) were injected onto an Ultra 2 capillary column (Conditions: carrier gas He; temperature: injector 200°C, oven 80°C (1 min) 80-290°C at 20°/min, 290°C (5 min)). The gas phase IR spectra of known alkaloids were entered into the the GC/IR library. The gas phase IR spectra of the unknown alkaloids were compared to library spectra by computer using a library search algorithm. The best computer match was visually inspected for confirmation of identity.

Isolation and Identification

When the sample was found to be a mixture of alkaloids and sufficient sample was available, the components were separated and the identity of the alkaloids was verified by spectral analysis including HREIMS, NMR, FTIR and by comparison with authentic samples. Many of the samples were isolated as perchlorate salts (B. $HClO_4$) or as picrate salts (B. pictate). The following pages list the individual 'L' numbered alkaloids, their components, and the methods of identification for each component.

L4 Previously Reported [40]:

Source: *L. complanatum* L.
MF: $C_{16}H_{27}N$
MW: 233
MP: 225°C (B. $HClO_4$)

Components identified in this study: L4-1 anhydrodihydrolycopodine

One compound was identified by GC/IR and by GC/MS, and shows a close match in the respective libraries to anhydrodihydrolycopodine. The HREIMS gives the molecular formula $C_{16}H_{25}N$, and is identical with anhydrodihydrolycopodine.

L13 Previously Reported [42]:

Sources: *L. tristachyum* Pursh
L. obscurum L.
L. clavatum L.
L. lucidulum Michx.
L. sabinaefolium Willd.
L. annotinum var. *acrifolium*
MF: $C_{16}H_{25}NO$
MW: 247
MP: 274°C (B. $HClO_4$)

Components identified in this study: L13-1 lycopodine

This compound was eventually identified as lycopodine by Marion and Manske (Dr. Marion's notebook 698, pg 57). Eleven samples of L13 were found in their sample collection, which were isolated from several *Lycopodium* species. Analysis by GC/IR and GC/MS showed that these samples were comprised mainly of lycopodine This confirms the earlier identification by Marion and Manske.

L14 Previously Reported [42]:
Source: *L. tristachyum* Pursh
MF: $C_{16}H_{25}N$
MW: 231
MP: 238°C (B. $HClO_4$)

Components identified in this study: L14-1 anhydrodihydrolycopodine

This compound was identified as anhydrodihydrolycopodine by Marion and Manske (Dr. Marion's notebook 698, pg 15). One compound was identified by GC/IR and by GC/MS, and close matches were found to anhydrodihydrolycopodine in the respective libraries The HREIMS gives the molecular formula $C_{16}H_{25}N$, and is identical with L4 and anhydrodihydrolycopodine. This confirms the identification work by Marion and Manske.

L15 Previously Reported [42]:
Source: *L. tristachyum* Pursh
MF: $C_{20}H_{31}NO_4$
MW: 349
MP: 231°C (B. $HClO_4$)

Components identified in this study: L15-1 lycopodine
L15-2 flabelliformine

Two compounds were identified by GC/MS and their mass spectra match those of lycopodine and flabelliformine. One major compound and a minor compound were observed using GC/IR spectroscopy, however, both compounds eluted almost simultaneously. The gas phase infrared spectrum of the major compound shows a very close library match to the spectrum of lycopodine, but it was not possible to unambiguously identify the minor compound. Thin Layer Chromatography (TLC) indicated a mixture of at least two compounds [R_f: 0.74, 0.29 (2% methanol in chloroform)]. When L15 (19 mg, B. $HClO_4$) was subject to chromatography over alumina (solvent system=1% MeOH/$CHCl_3$), two compounds were isolated and characterized (L15-1, 7.0 mg; L15-2, 3.7 mg). The first compound (R_f: 0.74) was identical with lycopodine (TLC, ^{1}H NMR, FTIR, HREIMS), and the second compound (R_f: 0.29) was identical with flabelliformine (TLC, ^{1}H NMR, FTIR, HREIMS). Because only lycopodine was identified using GC/IR, a pure sample of the second compound was subject to GC/IR. The results showed the presence of a single compound with an infrared spectrum identical with the library spectrum of flabelliformine.

L16 Previously Reported [43]:
Source: *L. obscurum* var. *dendroideum*
MF: $C_{16}H_{25}NO$
MW: 247
MP: 221°C (B. $HClO_4$)

Components identified in this study:
L16-1 lycopodine
L16-2 anhydrodihydrolycopodine
L16-3 acetylannofoline

Two samples of L16 were examined by GC/MS and GC/IR. One of the samples contains two compounds in a ratio of 1:1 for which library matches were made for lycopodine and anhydrodihydrolycopodine. The other sample contains three compounds in a ratio of 2:2:1, and library matches were made for lycopodine, anhydrodihydrolycopodine, and acetylannofoline, respectively.

L17 Previously Reported [43]:

Source: *L. obscurum* var. *dendroideum*
MF: $C_{18}H_{27}NO_3$
MW: 305
MP: 296°C (B. $HClO_4$)

Components identified in this study:
L17-1 acetylannofoline
L17-2 acetylacrifoline

Analysis by GC/MS and GC/IR showed the presence of two components with a ratio of 2:1. The first one shows close matches to the library spectrum of acetylannofoline, and the second one, to acetylacrifoline.

L18 Previously reported [44]:

Source: *L. clavatum* L.
MF: $C_{11}H_{19}NO$
MW: 181
MP: 195°C (B. picrate)

Components identified in this study:
L18-1 N,N-dimethylphlegmarine
L18-2 isomer of L18-1

Two compounds were observed using GC/MS and GC/IR. The GC/MS data showed two compounds both with a molecular weight of 278, and very similar mass fragmentation patterns. GC/IR data showed two compounds with very similar IR spectra. A close match was not found in the library spectra for either of the compounds.. TLC analysis showed only spot [R_f: 0.32 (5% MeOH/$CHCl_3$), R_f: 0.67 (10% MeOH/$CHCl_3$)]. After acid/base workup of the picrate derivative of L18 to obtain the free bases, L18 was subject to chromatography over alumina in attempts to separate the two compounds. These attempts were not successful and so a ^{1}H NMR spectrum was obtained for the mixture. One major and one minor compound were observed [major δ: 0.84 (d, 3H, J = 6.5 Hz), 2.16 ppm (s, 3H), 2.28 ppm (s,3H)] [minor δ: 0.92 (d,3H, J = 6.5Hz), 2.25 ppm (s,3H), 2.26

ppm (s,3H)]. HREIMS of the mixture revealed a molecular formula of $C_{18}H_{34}N_2$. The GC/IR spectra of each component show an absorption at ν=2780 cm^{-1}. These data suggests that the minor compound is N,N-dimethylphlegmarine, and that the major compound is probably an isomer of this compound.

L22 Previously reported [45]:

Source: *L. lucidulum* Michx.
MF: $C_{16}H_{27}NO$
MW: 249
MP: 108°C, 254°C (B. $HClO_4$)

Components identified in this study:

L22-1	lycopodine
L22-2	isolycodoline

Two compounds were observed using GC/MS (m/z=247, 263) and GC/IR. Close matches were found in the respective libraries correlating these two compounds to lycopodine and isolycodoline. TLC indicated the presence of two major compounds [R_f: 0.84, 0.71 (5% MeOH/$CHCl_3$)]. After acid / base workup of the perchlorate salt of L22 (105 mg) to obtain the free bases, L22 was subject to chromatography over alumina (solvent system=1% MeOH/$CHCl_3$), and a partial separation of the two compounds was obtained; the second compound (Rf=0.71) was obtained in pure form (L22-2, 43.1 mg). The first compound (Rf=0.84) was obtained in pure form (L22-1, 26 mg) after filtering and collecting the residue from the ethyl acetate washings of the extract obtained from the column. The first compound was identical with lycopodine (TLC, ^{1}H NMR, FTIR, HREIMS), and the second compound was identical with isolycodoline (TLC, ^{1}H NMR, ^{13}C NMR, FTIR, HREIMS).

L24 Previously Reported [45]: Source: *L. lucidulum* Michx.
MF: $C_{16}H_{25}NO$
MW: 247
MP: 278°C (B. $HClO_4$)

Components identified in this study: L24-1 lycopodine

One compound was observed by GC/MS and GC/IR, and was identical with the library spectra of lycopodine.

L25 Previously Reported [45]: Source: *L. lucidulum* Michx.
MF: $C_{16}H_{25}NO_2$
MW: 263
MP: 297°C (B. $HClO_4$)

Components identified in this study: L24-1 isolycodoline

One compound was identified by GC/IR and by GC/MS, and shows a very close match in the respective libraries to isolycodoline.

L26 Previously reported [46]: Source: *L. sabinaefolium* Willd.
MF: $C_{15}H_{25}NO$
MW: 235
MP: 171°C

Components identified in this study: L26-1 dihydrolycopodine

GC/MS and GC/IR both show the presence of only one compound (M/Z=249), and the libraries show a close match with dihydrolycopodine. TLC shows the presence of only one compound [R_f: 0.27 (2% MeOH/$CHCl_3$), R_f: 0.53 (5% MeOH/$CHCl_3$)]. The HREIMS data and the GC retention times of L26 are identical with those of dihydrolycopodine. The melting point of dihydrolycopodine (168°C) is also close to the reported melting point of L26. Because of small sample amounts, the quality of the ^{1}H NMR and FTIR spectra are poor and cannot be used to substantiate the assignment of L26.

L28 Previously reported[47]:
Source: *L. annotinum* var. *acrifolium*
MF: $C_{17}H_{27}NO_2$
MW: 277
MP: 211°C (B.$HClO_4$)

Components identified in this study:
L28-1 lycopodine
L28-2 5,15-oxidolycopodane

Two compounds were identified by GC/MS (m/z=247, 247). The compounds have similar but not identical mass fragmentation patterns. Two major compounds and one minor compound were observed using GC/IR. Good library matches of one of the compounds to lycopodine were obtained. The other compound(s) showed poor matches to library spectra. TLC indicated the presence of two major compounds [R_f: 0.72, 0.45 (2% MeOH/$CHCl_3$)]. The two major compounds were separated by subjecting L28 (65 mg) to column chromatography over alumina (solvent gradient: CH_2Cl_2 to 2% MeOH/CH_2Cl_2) (L28-1, 27 mg; L28-2, 24 mg). The second compound was found to decompose over a few days when exposed to air. The first compound was identical to lycopodine (TLC, ^{1}H NMR, FTIR, HREIMS). The second compound was identified as 5,15-oxidolycopodane. The identification of this compound is discussed separately.

L29 Previously Reported [47]:
Source: *L. annotinum* var. *acrifolium*
MF: $C_{16}H_{23}NO_2$
MW: 261
MP: 274°C (B. $HClO_4$)

Components identified in this study:
L24-1 acrifoline

One compound was identified by GC/IR and by GC/MS, and shows a close match in the respective libraries to acrifoline. This sample was previously identified in earlier studies in these laboratories by comparison (TLC, FTIR, HREIMS) with an authentic sample (T. Chua, 1986).

L31 Previously reported [47]:
Source: *L. annotinum* var. *acrifolium*
MF: $C_{20}H_{29}NO_4$
MW: 347
MP: 217°C ($B.HClO_4$)

Components identified in this study:
L31-1 O-acetyllofoline
L31-2 5,15-oxidolycopodane

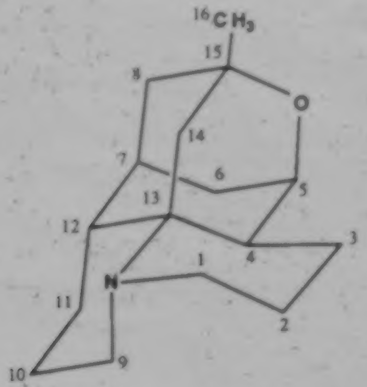
Two compounds were identified by GC/IR and GC/MS. The IR and MS spectra of the components are identical to acetyllofoline and 5,15-oxidolycopodane. TLC indicated the presence of at least two compounds [R_f: 0.66, 0.45 (2% $MeOH/CHCl_3$)]. After L31 (5.4 mg) was subject to chromatography over alumina (solvent = 1% $MeOH/CHCl_3$), one compound was isolated in pure form (L31-1, 4.4 mg). This compound was identical to O-acetyllofoline (^1H NMR, ^{13}C NMR, FTIR, HREIMS). The other compound (L31-2, 0.9 mg) was not isolated in pure form, and the sample amount was very small but the spectral data gathered suggests the presence of 5,15-oxidolycopodane (TLC, HREIMS, ^1H NMR, FTIR, GC).

L35 Previously reported [49]:
Source: *L. densum* Labill.
MF: $C_{14}H_{21}NO$
MW: 219
MP: 133°C

Components identified in this study: L35-1 lycopodine
L35-2 lycodoline
L35-3 flabelliformine

Three compounds were observed by GC/MS and by GC/IR and display library matches for lycopodine, flabelliformine and lycodoline. TLC indicated the presence of at least three compounds [R_f: 0.79, 0.53, 0.44 (5% MeOH/$CHCl_3$)]. One major and two minor compounds were isolated following column chromatography over alumina (L35, 47 mg) [solvent gradient: hexane/ethyl acetate (3:1) to ethyl acetate to ethyl acetate/methanol (10:1)]. The first compound (L35-1, 27 mg) was identical to lycopodine (TLC, ^{1}H NMR, ^{13}C NMR, HREIMS, FTIR). The second compound (L35-2, 3.1 mg) was similar to lycodoline (TLC, ^{1}H NMR, HREIMS FTIR), and the third compound was similar to flabelliformine (L35-3, 3.2 mg) (TLC, ^{1}H NMR, HREIMS, FTIR).

5,15-oxidolycopodane



Alkaloid L28 (*L. annotinum*) was chromatographed (2% CH_2Cl_2 / CH_3OH) to give two compounds. The less polar constituent was identified as lycopodine by comparison with an authentic sample. The more polar component was identified as oxidolycopodane [71]: **$[\alpha]_D$** = 51° (*c* 0.019, CH_3OH); **FTIR** ($CHCl_3$ cast): 2964, 2925, 2860, 1442, 1132, 1110 cm^{-1}; **^{1}H NMR** ($CDCl_3$): δ 3.63 (1H, m, H-5), 3.36 (1H td, *J* = 3.5, 14.5 Hz, H-1e), 3.19 (1H, td, *J* = 3.5, 12.0 Hz, H-9e), 2.64 (1H, dd, *J* = 2.0, 12.0 Hz, H-14a), 2.53 (1H, dm, *J* = 12.5 Hz, H-1a), 2.05 (1H, m, H-4), 1.97 (1H, m, H-2a), 1.89 (2H, m, H-3a, H-6a), 1.78 (1H, m, H-10e), 1.73 (1H, ddd, *J* =2.5, 2.5, 12.0 Hz, H-11), 1.70 (2H, m) 1.67 (1H, m, H-10), 1.63 (1H, m), 1.60 (1H, m), 1.55 (1H, ddd, *J* = 2.0, 4.0, 12.0 Hz, H-8a), 1.47 (1H, m, H-3e), 1.40 (1H, m), 1.27 (1H, dm, *J* = 13Hz, H-2a), 1.15 (3H, s, H-16), 1.06 (1H, d, *J* = 12.0Hz, H-14e); **^{13}C NMR** ($CDCl_3$, APT): δ 74.3 (d, C-5), 72.3 (s, C-15), 54.3 (s, C-13), 46.8 (d, C-12), 47.4 (t, C-9), 47.0 (t, C-1), 43.9 (t, C-14), 42.1 (t, C-8), 33.9 (d, C-7), 31.4 (d, C-4), 31.0 (t, C-6), 29.2 (q, C-16), 26.5 (t, C-10), 25.6 (t, C-11), 24.6 (t, C-3), 18.6 (t, C-); **HREIMS** *m/z* (relative intensity): 247.1944 found, 247.1936 calcd for $C_{16}H_{25}NO$ (47), 191 (10), 190 (60), 176 (10), 175 (15), 174 ($C_{12}H_{16}N$, 100), 163 (17), 146 (10).

Transformation of dihydrolycopodine to 5,15-Oxidolycopodine

Lead tetraacetate (100 mg) and iodine (cat amount) were added to a solution of dihydrolycopodine (26 mg) in dichloromethane (5 mL). The reaction mixture was irradiated for 16 hours using a 75W light bulb. Water (10 mL) was added and the layers were separated. The aqueous fraction was extracted several times with dichloromethane, then basified (aq. ammonia) and extracted exhaustively with dichloromethane. The dichloro-methane layer was subjected to acid-base workup in the usual way. The resultant dichloromethane fraction was concentrated to give an oil (14.5 mg) which was shown to be a mixture of 3 components (TLC). Chromatography over alumina gave dihydrolycopodine (5.4 mg) and 5,15-oxidolycopodine (8.0 mg), which had identical ^{1}H and ^{13}C NMR, IR, and HREIMS spectra data to that reported above. The third component decomposed before it was fully characterized.

Analysis of an extract of *L. clavatum.*

The analysis of L18 suggests that N,N-dimethylphlegmarine was present in *L. clavatum* L. Because this is the first reported case of this compound found in *L. clavatum*, a methanol extract of *L. clavatum*, available from previous work in these laboratories, was examined for the presence of this compound. The crude alkaloids (1.54 grams) were extracted from 215 grams of the methanol extract by acid / base extraction. The entire sample was chromatographed over alumina, and eluted with 2% to 5% MeOH in $CHCl_3$. Fractions containing compounds with similar R_f values by TLC were combined. The fraction containing compounds with R_f's close to the R_f value for L18 was examined by GC/IR but none of the infrared spectra were similar to that of N,N-dimethylphlegmarine.

Table 24: Compounds in the GC/MS and GC/IR library*

Molecular Weight	Molecular Formula	Name	Molecular Weight	Molecular Formula	Name
207	$C_{13}H_{21}NO$	luciduline	274	$C_{17}H_{26}N_2$	dihydroobscurinine
231	$C_{16}H_{25}N$	anhydrodihydrolycopodine*	274	$C_{17}H_{26}N_2O$	sauroxine
242	$C_{16}H_{22}N_2$	lycodine	275	$C_{16}H_{21}NO_3$	annotine
242	$C_{15}H_{18}N_2O$	selagine	275	$C_{16}H_{21}NO_3$	annotinine
245	$C_{16}H_{23}NO$	fawcettidine	278	$C_{16}H_{26}N_2O_2$	lycocernuine
247	$C_{16}H_{25}NO$	lycopodine	279	$C_{16}H_{25}NO_3$	alopecuridine
248	$C_{16}H_{28}N$	dihydrodeoxycernuine	291	$C_{18}H_{29}NO_3$	clavine
249	$C_{16}H_{27}NO$	dihydrolycopodine	291	$C_{17}H_{25}NO_3$	lyconnotine
258	$C_{16}H_{22}N_2O$	des-N-methyl-β-obscurine	303	$C_{18}H_{25}NO_3$	acetylacrifoline
260	$C_{16}H_{24}N_2O$	des-N-methyl-α-obscurine	305	$C_{18}H_{27}NO_3$	acetylannofoline*
261	$C_{16}H_{23}NO_2$	acrifoline	305	$C_{18}H_{27}NO_3$	dehydro-α-lofoline
261	$C_{16}H_{23}NO_2$	serratidine	305	$C_{18}H_{27}NO_3$	dehydrofawcettiine
262	$C_{16}H_{26}N_2O$	cernuine	305	$C_{18}H_{27}NO_3$	dihydro-O-acetylacrifoline
263	$C_{16}H_{25}NO_2$	clavolonine	307	$C_{18}H_{29}NO_3$	α-lofoline
263	$C_{16}H_{25}NO_2$	flabelliformine	307	$C_{18}H_{29}NO_3$	fawcettiine (β-lofoline)
263	$C_{16}H_{25}NO_2$	isolycodoline	307	$C_{18}H_{29}NO_3$	lycoclavine
263	$C_{16}H_{25}NO_2$	lucidioline	320	$C_{18}H_{28}N_2O_3$	acetyllycocernuine*
263	$C_{16}H_{25}NO_2$	lycodoline	321	$C_{18}H_{28}NO_4$	N-acetylalopecuridine
263	$C_{16}H_{25}NO_2$	lycofoline	323	$C_{18}H_{29}NO_4$	lycofawcine
264	$C_{16}H_{28}N_2O$	dihydrodeoxylycocernuine	333	$C_{19}H_{27}NO_5$	acetylannopodine
265	$C_{16}H_{27}NO_3$	dihydroclavolonine	349	$C_{20}H_{31}NO_4$	acetyllofoline
272	$C_{17}H_{24}N_2$	isoobscurinine*	349	$C_{20}H_{31}NO_4$	acetylfawcettiine
272	$C_{17}H_{24}N_2$	obscurinine	349	$C_{20}H_{31}NO_4$	acetyllycoclavine
274	$C_{16}H_{26}N_2O$	α-obscurine	367	$C_{23}H_{29}NO_3$	alopecurine

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* Alkaloids not present in the GC/MS library.

EXPERIMENTAL - *Lycopodium meridionale*

Isolation of LM-1, Lycocernuine and LM-3

The plant material for this work was collected in Guyana by Claude A. Persaud in 1986. This plant is described as creeping club moss from savanna growing on poorly drained white sand soil, located near the Ekereku River. Some of this material was deposited in the University of Alberta Herbarium where its identity as *Lycopodium meridionale* was confirmed. The specimens were given accession numbers 92374 and 92375.

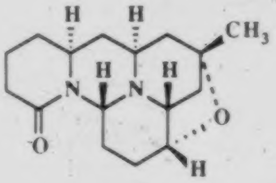
Dried, ground *Lycopodium meridionale* (847 grams) was divided into three approximately equal portions and each portion was suspended in 4 liters 2% w/v aqueous tartaric acid for 24 hours. Each suspension was then filtered and the partially extracted plant material further extracted in the same manner with, successively, 2 liters of 2% aqueous tartaric acid, and 2 liters of water, each for 24 hours. The aqueous extracts were combined and concentrated under reduced pressure to 1/10 of the original volume, made basic to pH 9 with conc. NH_4OH, and extracted with diethyl ether and then with dichloromethane. The crude organic extracts were combined, dissolved in 2% aqueous tartaric acid and extracted with dichloromethane. The aqueous layer was made strongly basic with NH_4OH and extracted with dichloromethane. Evaporation of the dichloromethane yield 4.99 grams of crude bases.

A sample of the crude bases (110 mg) was chromatographed over alumina. Elution with 1% CH_3OH in CH_2Cl_2 gave successively, LM-1 (21.8 mg), LM-2 (34.7 mg), and LM-3 (33.1 mg). Each base was further purified by passage through a disposable pipette packed with alumina. Elution with CH_2Cl_2 followed by 2% CH_3OH in CH_2Cl_2, while monitoring closely by TLC, yielded the purified bases.

Lycocernuine (LM-2), which was required in larger amounts as starting material for chemical transformations, could also be isolated in pure form from the crude alkaloid extract by either fractional crystallization [36a] or centrifugal counter-current chromatography [84]. Thus, the crude alkaloid extract was dissolved in hot acetone, the solution allowed to cool, and the crystals that formed were filtered off and recrystallized to give pure (TLC, ^{1}H NMR) lycocernuine. Approximately 300 mg of lycocernuine was obtained in this way from the crude alkaloid extract. Alternately, centrifugal counter-current chromatography, using ethyl acetate - water, afforded approximately 100 mg of lycocernuine from 700 mg of extract [mobile phase - ethyl acetate; centrifuge spinning rate - 800 rpm; solvent flow rate - 3 ml/min; 200-250 mg extract per run; lycocernuine elution time - 110 to 150 minutes].

Characterization:

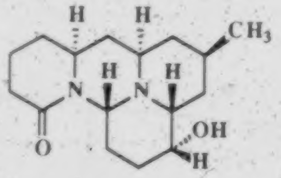
LM-1 (12, 15-oxidocernuine, "lycomeridine"):



LM-1 was isolated as an oil which solidified upon standing, and melted at 89-92°C: $[\alpha]_D$ -30.3° (*c* 0.190, CH_3OH); **TLC** R_f 0.75 (2% CH_3OH-$CHCl_3$), 0.84 (10% CH_3OH-$CHCl_3$); **FTIR** ($CHCl_3$ cast): 2959s, 2928s, 1640s, 1436m, 1413m, 1332w, 1298w, 1136w cm^{-1}; **^{1}H NMR ($CDCl_3$)**: (no D_2O exchangeable hydrogens) δ 5.54 (1H, dd, *J* = 10, 5.0 Hz), 3.99 (1H, ddd, *J* = 5.0, 2.0, 2.0 Hz), 3.58 (2H,m), 3.32 (1H, dd, *J* = 3.0, 3.0 Hz), 2.44 (1H,dddd, *J* = 16, 4.0, 2.5, 2.5 Hz), 2.35 (1H, ddd, *J* = 16, 11, 6.0 Hz), 1.34 (3H, s), 2.10-1.20 (14H, m); **(d6-benzene)**: δ 5.77 (1H, dd, *J* = 11, 5 Hz), 3.63 (1H, ddd, *J* = 5.0, 2.0, 2.0 Hz), 3.36 (1H, dddd, *J* = 11, 11, 6.0, 2.0

Hz), 2.99 (1H,m - decoupling at 1.0 --> dd, J = 8.2, 3.2 Hz), 2.85 (1H, dd, J =2.0, 2.0 Hz), 2.36 (1H, dddd, J = 17, 5.0, 2.5, 2.5 Hz), 2.15-1.90 (3H, m), 1.56 (1H, dddd, J = 15, 9.5, 9.5, 6.0 Hz), 1.49 (1H, d, J = 11 Hz), 1.45 (1H, m), 1.39 (1H, ddd, J = 11, 3.2, 2.8 Hz), 1.24 (3H, s), 1.40-1.00 (8H, m). **^{13}C NMR** ($CDCl_3$, APT): δ 168.8(s), 80.8(t), 74.8(d), 66.3(d), 62.3(d), 53.0(d), 48.4(d), 44.3(t), 43.1(t), 39.1(t), 32.9(t), 30.8(t), 25.7(q), 24.9(t), 20.0(t), 13.9(t); **CIMS** (NH_3): *m/z* 277 (100%) $[M+H]^+$; **HREIMS:** *m/z* (relative intensity): 276.1835 found, 276.1838 calcd for $C_{16}H_{24}N_2O_2$ (100), 261 (33.3), 233 (46.7), 219 (11.2), 209 (37.6), 205 (12.9), 165 (46.7), 164 (19.5), 149 (28.5), 122 (14.5), 65(49.3), 55 (10.7), 55 (16.1).

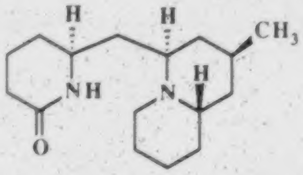
LM-2 (lycocernuine) [35]:



LM-2 crystallized from acetone and melted at 229-230°C, and the spectral data is identical with that found for lycocernuine: **TLC** R_f 0.53 (2% CH_3OH-$CHCl_3$), 0.78 (10% CH_3OH-$CHCl_3$); **FTIR** ($CHCl_3$ cast): 3400m, 2948s, 2885s, 1621s, 1442m, 1418w, 1340w, 1300w, 1258w, 1223w, 995w, 752w cm^{-1}; **^{1}H NMR ($CDCl_3$):** δ 5.49 (1H, dd, J = 12.0, 2.5 Hz), 3.85 (1H, dd, J = 2.5, 2.5 Hz), 3.55 (2H, m), 3.00 (1H, d, J = 6.5 Hz), 2.47 (1H, dm, J = 17.0 Hz), 2.45 (1H,m), 2.37 (1H, dd, J = 17.0, 5.0 Hz), 2.31 (1H,m), 1.23 (1H, dm, J = 13.0 Hz), 2.1 - 1.3 (10H), 1.22 (1H, s, D_2O exchangeable), 1.17 (1H, q, J = 12.0 Hz), 0.91 (3H, d, J = 6.5 Hz), 0.87 (1H, q,J = 12.0 Hz); **(benzene-d6)**: 5.75 (1H, dd, J = 12.0, 2.5 Hz), 3.36 (1H, dddd, J = 5.5, 5.5, 3.0, 3.0 Hz), 3.14 (1H, dd, J = 2.5, 2.5 Hz), 3.06 (1H, m), 2.60 (1H, d, J = 6.5 Hz), 2.34 (1H, dm, J = 17.0 Hz), 2.30 (1H, m), 2.15 (1H,dd, J = 17.0, 5.0 Hz), 2.10 (1H, m), 2.0-1.0 (12H), 1.00 (1H, q, J = 12.0 Hz), 0.89 (3H, d, J = 6.5 Hz), 0.85

(1H, q, J = 12.0 Hz); **^{13}C NMR** ($CDCl_3$, APT): δ 168.4(s), 71.0(d), 67.3(d), 58.6(d), 51.0(d), 49.0(d), 41.8(t) (2 carbons), 38.0(t), 33.8(t), 33.2(t), 30.5(t), 26.4(q), 23.0(d), 19.3(t), 15.9(t); **HREIMS:** *m/z* (relative intensity): 278.1995 found, 278.1994 calcd for $C_{16}H_{26}N_2O_2$ (50), 261 (16), 249 (22), 220 (70), 219 (100), 165 (22.8), 152 (13.8), 110 (7.8).

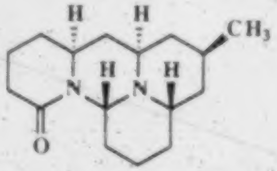
LM-3 (13-epidihydroallocernuine):



LM-3 was isolated as an oil which solidified upon standing, and melted at 81-82°C: $[\alpha]_D$ +46.3° (*c* 0.201,MeOH); **TLC R$_f$** 0.19 (2% CH_3OH-$CHCl_3$), 0.57 (10% CH_3OH-$CHCl_3$); **FTIR ($CHCl_3$ cast):** 3215m, 3018m, 2926s, 2865s, 1664s, 1455w, 1445w, 1408w, 1343w, 1308w, 751w cm^{-1}; **($CHCl_3$ soln):** 3400 sharp, 2914s,1646s, 1450w, 1340m cm^{-1}; **^{1}H NMR** ($CDCl_3$): δ 6.65 (1H, brs, D_2O exchangeable), 3.46 (1H, dddd, J = 9.5, 9.0, 3.5, 3.5 Hz), 3.15 (1H, ddd, J = 12.0, 4.0, 3.0 Hz), 3.04 (1H, m), 2.92 (1H, m), 2.61 (1H, ddd, J = 12.0, 10.0, 3.2 Hz), 2.35 (1H, dm, J = 17 Hz), 2.25 (1H, ddd, J = 17.0, 10.5, 6.0 Hz), 1.78 (1H, ddd, J = 13.5, 9.0, 4.5 Hz), 1.32 (1H, ddd, J = 13.5, 7.5, 3.5 Hz), 0.88 (3H, d, J = 6.0 Hz), 0.84 (1H, ddd, J = 12.0, 12.0, 10.0 Hz), 1.90-1.10 (14H); **^{13}C NMR** ($CDCl_3$, APT): δ 171.5 (s), 54.7 (d, br), 51.2 (d, br, 2 carbons), 49.5 (t), 40.4 (t), 38.9 (t, br), 37.1 (t, br), 31.3 (t), 30.1 (t), 25.7 (d), 25.5 (t, br), 24.2 (t, br), 22.4 (q), 20.6 (t, br), 20.1 (t); **CIMS** (NH_3): *m/z* 265 (100%) $[M+H]^+$, 152 (12 %); **HREIMS:** *m/z* (relative intensity): 264.2199 found, 264.2202 calcd for $C_{16}H_{28}N_2O_2$ (6.9), 166 (5.7), 153 (14.1), 152 (100), 110 (9.5), 84 (3.8), 55 (4.8).

The ^{1}H NMR and ^{13}C NMR spectrums were also acquired at 55°C: **^{1}H NMR** ($CDCl_3$, 55°C): δ 3.49 (1H, ddd, J = 13.0, 9.0, 3.5 Hz), 3.13 (1H, dm, J = 13.0 Hz), 3.08 (1H, m), 2.91 (1H, m), 2.63 (1H, ddd, J = 10.0, 10.0, 3.5 Hz), 2.38 (1H, dddd, J = 16.5, 6.0, 3.5, 2.0 Hz), 2.27 (1H, ddd, J = 16.5, 10.0, 6.0 Hz), 0.80 (3H, d, J = 6.5 Hz); **^{13}C NMR** ($CDCl_3$, 55°C): δ 172.0, 53.9 (br), 52.1 (br), 51.6, 49.7, 40.6, 38.6, 36.7 (br), 31.3, 30.2, 26.0, 25.7, 23.9, 22.4, 21.1, 20.1 .

Cernuine [35]:



Cernuine was not isolated from *L. meridionale*, although it was detected by GC/IR. Because of its structural similarity to the major compounds isolated from *L. meridionale*, previously unreported spectral data was collected for cernuine to aid in the assignment the spectral data of other compounds discusssed in this thesis. Cernuine was obtained from our collection of *Lycopodium* alkaloids. **^{1}H NMR** ($CDCl_3$): δ 5.43 (1H, dd, J = 11.5, 3.0 Hz), 3.44 (1H, m), 3.07 (1H, m), 3.04 (1H, dddd, J = 10.5, 10.5, 2.5, 2.5 Hz), 2.36 (1H, dddd, J = 17.0, 5.5, 4.0, 2.0 Hz), 2.26 (1H, ddd, J = 17.0, 11.0, 6.0 Hz), 1.18 (1H, q, J = 11.5 Hz), 0.99 (1H, dm, J = 12.0 Hz), 0.82 (3H, J = 6.5 Hz), 0.80 (1H, q, J = 11.5 Hz); **^{13}C NMR** ($CDCl_3$, APT): δ 168.1 (s), 67.2 (d), 57.5 (d), 50.6 (d), 46.1 (d), 42.0 (t), 41.2 (t), 39.3 (t), 33.0 (t), 30.5 (t), 25.3 (d), 24.5 (t), 22.3 (t), 22.2 (q), 20.0 (t), 19.3 (t).

Reactions:

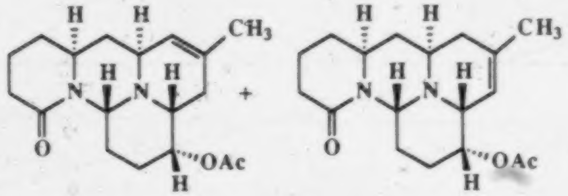
Attempted acetylation of LM-1:

LM-1(4.2 mg) was dissolved in acetic anhydride (1 ml) and pyridine (1 drop) and a few small crystals of dimethyaninopyridine were added and the reaction was left for 3 days at room temperature. Water was added to the reaction mixture and dilute ammonia was added to reach a pH of 9. The solution was extracted with dichloromethane, dried and evaporated. Analysis by TLC, ^{1}H NMR and HREIMS indicated the product was unreacted LM-1.

Ether cleavage of LM-1:

Boron trifloride - diethyl etherate (0.5 ml, freshly distilled) was added to a solution of LM-1 (30 mg) in acetic anhydride (4.0 ml) and the mixture was refluxed gently for 1 hour. The solution was then concentrated *in vacuo* and water was added to the residue. The aqueous mixture was extracted with dichloromethane, and the extract washed with 2% v/v hydrochloric acid. The two aqueous phases were combined, basified to pH 9 with conc. aqueous ammonia and extracted with dichloromethane. Evaporation of the dried extract gave an oil (19.2 mg) which was a chromatographically inseparable mixture of two compounds (^{1}H NMR, ^{13}C NMR). When the reaction was performed at room temperature for 24 hours a similar (TLC, ^{1}H NMR, ^{13}C NMR) mixture of the two inseparable compounds were obtained. These isomeric products were labeled LM-1-A.

LM-1-A:

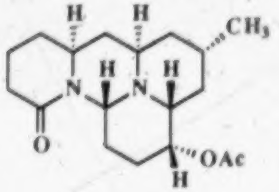


TLC: R_f 0.54 (2% CH_3OH-$CHCl_3$); **FTIR** ($CHCl_3$ cast): 2939m, 2911m, 1735s, 1690w, 1641s, 1437m, 1415m, 1372m, 1337m, 1299m, 1235s, 1175m, 1020m, 840m cm^{-1}; **^{1}H NMR** ($CDCl_3$) **Major isomer:** δ 5.62 (1H, dd, *J* = 9.0, 2.5 Hz), 5.22 (1H, s), 4.93 (1H, dd, *J* = 2.5, 2.0 Hz), 3.88 (1H, d, *J* = 12.0 Hz), 3.58 (2H, m), 1.96 (3H, s), 1.65 (3H, s); **Minor isomer:** δ 5.58 (1H, dd, *J* = 9.0, 2.5 Hz), 5.17 (1H,s), 4.96 (1H, dd, *J* = 2.5, 2.0 Hz), 3.58 (2H, m), 3.29 (1H, d, *J* = 8.5 Hz), 1.95 (3H, s), 1.62 (3H, s); **^{13}C NMR** ($CDCl_3$) **Major isomer:** δ 169.8, 168.3, 132.9, 119.2, 68.7, 65.9, 59.0, 50.4, 45.8, 41.8, 37.4, 33.0, 30.3, 28.8, 22.8, 21.2, 19.0, 16.6; **Minor isomer:** δ 170.3, 168.5, 130.9, 121.7, 71.0, 67.2, 56.7, 52.5, 48.9, 40.2, 37.4, 32.5, 30.5, 29.4, 22.7, 21.2, 19.6, 15.6; **HREIMS:** *m/z* (relative intensity): 318.1933 found, 318.1944 calcd for $C_{16}H_{26}N_2O_3$ (68.5), 303 (8.6), 275 (29.0), 259 (72.7), 247 (12.2), 231 (8.9), 217 (100), 147 (22.6), 94 (26.9); **GC/IR Major isomer:** 2950s, 1755s, 1675s, 1412m, 1295m, 1229s, 1128w, 1016w, 841w cm^{-1}; **Minor isomer:** 2949s, 1757s, 1678s, 1415m, 1294m, 1229s, 1135w, 977w, 851w cm^{-1}.

Hydrogenation of LM-1-A:

A suspension of LM-1-A (20.0 mg) in 95% ethanol (1 ml) containing 5% palladium on carbon (20 mg) was stirred under an atmosphere of hydrogen for 3 days. The mixture was filtered and the catalyst was washed with hot 95% ethanol. The combined filtrates were evaporated and the product was chromatographed on alumina. Elution with 2% methanol in dichloromethane gave 15-epiacetyllycocernuine [33] (16 mg) which could not be induced to crystallize.This compound was designated LM-1-A-H.

LM-1-A-H (O-acetylepilycocernuine):



TLC R$_f$ 0.59 (2% CH_3OH-$CHCl_3$); **FTIR** ($CHCl_3$ cast): 2944s, 2929s, 2865s, 1737s, 1641s, 1459m, 1438m, 1416m, 1372m, 1339m, 1299m, 1271m, 1238s, 1207m, 1143m, 1127m, 1120m, 1018m, 751m cm^{-1}; **^{1}H NMR** ($CDCl_3$): δ 5.42 (1H, dd, J = 11.5, 2.5 Hz), 4.68 (1H, d, J = 2.5 Hz), 3.47 (2H, m), 3.28 (1 H, ddd, J = 10.5, 6.0, 2.0 Hz), 2.43 (1H, dm, J = 17.0 Hz), 2.33, (1H, ddd, J = 17.0, 11.5, 5.5 Hz), 2.12 (3H, s), 1.58 (1H, ddd, J = 12.5, 2.5, 2.5 Hz), 0.87 (3H, d, J = 6.5 Hz); **^{13}C NMR** ($CDCl_3$, APT): δ 170.5(s), 168.4(s), 70.7(d), 66.9(d), 57.0(d), 51.7(d), 46.3(d), 42.9 (t, two carbons), 38.7(t), 33.6(t), 33.0(t), 30.4(t), 29.0(t), 22.9(q), 21.5(q), 19.4(t), 16.0(t); **HREIMS:** *m/z* (relative intensity): 320.2096 found, 320.2100 calcd for $C_{16}H_{28}N_2O_3$ (29.0), 277 (11.9), 261 (81.6), 249 (32.3), 233 (10.0), 219 (100), 162 (4.8), 149 (10.7); **GC/IR:** 2947s, 2877m, 1758s, 1679s, 1416m, 1371m, 1280m, 1233s, 1140w, 1020w cm^{-1}.

Acid Hydrolysis of LM-1-A:

LM-1-A (20 mg) was dissolved in 5% v/v hydrochloric acid (2 ml) and heated on a steam bath for 17 hours. The solution was allowed to cool and adjusted to pH 10 with 5% w/v sodium hydroxide. The aqueous mixture was extracted with dichloromethane and the dried extracts evaporated to an oil (15 mg). Analysis by GC/IR showed the presence of 5 compounds. A library search showed that two compounds have IR spectra similar to but not identical with lycocernuine, and 3 compounds have IR spectra similar but not identical to acetyllycocernuine.

The above mixture of compounds (14 mg) was dissolved in 5% v/v hydrochloric acid (2 ml) and heated on a steam bath for a further 67 hours. The solution was worked up as above to give 7.3 mg of product. Analysis by GC/IR showed the presence of 1 major and 2 minor compounds. A library search showed that the major compound and one of the minor compounds have IR spectra similar but not identical to lycocernuine. The other minor compound has an IR spectrum identical to LM-1.

Attempted oxidation of lycocernuine to LM-1:

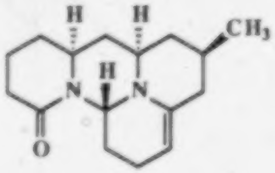
A number of attempts were made to oxidize lycocernuine to LM-1 using lead tetraacetate. One unsuccessful attempt involved refluxing a solution of lycocernuine and lead tetraacetate in dichloromethane for 3 hours. Another unsuccessful attempt involved refluxing a solution of lycocernuine and lead tetraacetate in chloroform for 3.5 hours. The last attempt involved irradiating a solution of lycocernuine, lead tetraacetate and iodine with 75 watt incandescent light bulb for 12 hours at room temperature. Analysis by ^{1}H NMR, GC/IR and TLC showed that lycocernuine was not oxidized to LM-1. The ^{1}H NMR spectra for all the products of each of the above oxidation attempts show a doublet methyl group at δ 0.91, and no methyl singlet corresponding to that in the spectrum of LM-1.

Formation of anhydrolycocernuine [36a]:

Methanesulfonyl chloride (0.75 ml) was added slowly to a solution of lycocernuine (65 mg, 0.23 mmol) in pyridine (4 ml) cooled (0°C) for 72 hours, then 5% w/v sodium hydroxide (20 ml) was added and the solution extracted with dichloromethane. The extract was dissolved in methanol (3 ml) and added to a solution of sodium methoxide (from 200mg sodium) in methanol (3 ml). After being refluxed for 3 hours, the solution was concentrated *in vacuo*, water added, and the mixture extracted with dichloromethane.

Evaporation of the dried dichloromethane extract yielded crude anhydrolycocernuine. Sublimation (house vacuum, heat gun) gave pure anhydrolycocernuine as a white powder (46 mg, 0.18 mmol, 77%).

Anhydrolycocernuine:

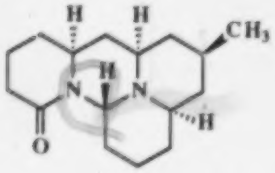


The white powder of anhydrolycocernuine melted at 139-140°C: **TLC** R_f 0.76 (2% CH_3OH-$CHCl_3$); **^{1}H NMR** ($CDCl_3$): δ 5.46 (1H, dd, *J* = 11.5, 1.5 Hz), 4.70 (1H, d, *J* = 4.5 Hz), 3.36 (1H, m), 2.64 (1H, dddd, *J* = 11.0, 11.0, 3.0, 2.5 Hz), 2.39-2.14 (3H, m), 1.76 (1H, dd, *J* = 11.5, 5.5 Hz), 1.61 (1H, ddd, *J* = 11.5, 2.5, 2.5 Hz), 1.53 (1H, ddd, *J* = 12.5, 3.0, 3.0 Hz), 1.06 (1H, q, *J* = 12.5 Hz), 0.82 (3H, d, *J* = 6.5 Hz); **^{13}C NMR** ($CDCl_3$, APT): δ 168.3 (s), 145.0 (s), 103.8 (d), 64.5 (d), 54.1 (d), 50.2 (d), 40.7 (t), 40.3 (t), 39.4 (t), 32.4 (t), 31.6 (d), 29.3 (t), 23.4 (t), 21.1 (q), 18.7 (t), 18.1 (t); **HREIMS:** *m/z* (relative intensity): 260.1879 found, 260.1888 calcd for $C_{16}H_{24}N_2O$ (52.0), 259 (49.8), 245 (25.9), 162 (40.9), 149 (100), 134 (14.0), 120 (13.0), 107 (10.5).

Formation of allocernuine [36a]:

A mixture of anhydrolycocernuine (12 mg) and platinum oxide (20 mg) in methanol (3ml) was stirred under a hydrogen atmosphere for 4 hours at room temperature and atmospheric pressure. The catalyst was filtered off and washed successively with methanol and dichloromethane. Evaporation of the combined filtrates gave an oil (10 mg) pure by TLC and ^{1}H NMR (when longer hydrogenation times (8 hours) were used, mixtures of products were obtained).

Allocernuine:



Tlc R_f 0.90 (2% CH_3OH-$CHCl_3$); **FTIR** ($CHCL_3$ cast): 2924s, 2866s, 1639s, 1448m, 1410m, 1197m, 1100m cm^{-1}; **^{1}H NMR** ($CDCl_3$): δ 4.93 (1H, dd, J = 10.0, 2.5 Hz), 3.96 (1H, dddd, J = 12.5, 6.5, 4.0, 4.0 Hz), 3.02 (2H, m), 2.33 (2H, m), 2.19 (1H, bd, J = 13.0 Hz), 2.11 (1H, ddd, J = 13.0, 11.0, 4.0 Hz), 0.89 (3H, d, J = 5.0 Hz); **^{13}C NMR** ($CDCl_3$, APT): δ 168.8 (s), 63.5 (d), 58.3 (d), 55.2 (d), 46.5 (d), 38.4 (t), 35.5 (t), 33.2 (t), 32.2 (d), 31.0 (t), 30.1 (t), 30.0 (t), 27.7 (t), 22.1 (q), 19.0 (t), 17.0(t); **HREIMS:** *m/z* (relative intensity): 262.2039 found, 262.2045 calcd for $C_{16}H_{26}N_2O$ (38.9), 247 (6.0), 234 (21.2), 233 (100), 219 (23.9), 205 (4.8), 191 (5.0), 149 (8.5).

Similar hydrogenation of anhydrolycocernuine over 5% palladium on carbon in ethyl acetate also gave allocernuine.

Formation of Dihydroallocernuine [36a]:

a) From anhydrolycocernuine:

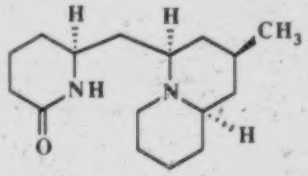
A suspension of anhydrolycocernuine (90 mg) in methanol containing 5% palladium on carbon (90 mg) was stirred under an atmosphere of hydrogen. After 24 hours, TLC analysis of the mixture showed one major spot close to the starting material,and one very weak spot. The reaction mixture was hydrogenated for another 24 hours, the mixture was filtered, and the catalyst was washed with hot methanol. After column chromatography over alumina, 16 mg of dihydroallocernuine was obtained as an oil.

Similar hydrogenation of anhydrolycocernuine over platinum oxide in methanol for periods longer than 4 hours gave mixtures of products, one of which is dihydrallocernuine. For example, from 25 mg of anhydrolycocernuine 2 mg of dihydroallocernuine was obtained after 8 hours of hydrogenation. The major product (7 mg) was allocernuine (see above for allocernuine).

b) From allocernuine:

A suspension of allocernuine (7mg) in methanol (6 ml) containing sodium borohydride (19 mg) and potassium carbonate (50 mg) was refluxed for 17 hours. Water (30 ml) was added and the solution was extracted with dichloromethane. Chromatography of the dichloromethane extract over alumina gave dihydroallocernuine (5 mg) as an oil.

Dihydroallocernuine:



Tlc R_f 0.35 (2% CH_3OH-$CHCl_3$); **FTIR** ($CHCL_3$ cast): 3211m, 3080m, 2925s, 2867s, 2790s, 1666s, 1445m, 1409m, 1387m, 1372m, 1347m, 1331m, 1307m, 1391m, 1181m, 1162m, 1101m, 1081m, 751m cm^{-1}; ^{1}**H NMR** ($CDCl_3$): δ 5.92 (1H, vbs), 3.50 (1H, ddd, J = 13.0, 9.0, 3.5 Hz), 3.14 (1H,s), 2.39 (1H, dm, J = 18.0 Hz), 2.28 (1H, ddd, J = 18.0, 10.5, 6.0 Hz), 1.03 (1H, q, J = 12 Hz), 0.89 (3H, d, J = 6.0 Hz); ^{13}**C NMR** ($CDCl_3$): δ 172.0, 62.6 (br), 60.2 (br), 51.1 (br, 2 carbons), 40.8 (br, 3 carbons), 33.6 (br), 31.3, 30.6, 29.8, 26.3, 24.0 (br), 22.0, 19.9; **HREIMS:** *m/z* (relative intensity): 264.2203 found, 264.2202 calcd for $C_{16}H_{28}N_2O$ (6.7), 166 (5.7), 152 (100), 149 (4.0), 110 (14.3), 98 (3.2), 84 (5.7).

The ^{1}H NMR and ^{13}C NMR spectra of dihydroallocernuine were obtained also at higher temperatures. **^{1}H NMR** (CDCl$_3$, 55°C): Signals sharpen. **^{13}C NMR** (CDCl$_3$, 55°C): δ 171.9, 62.6 (br), 60.3, 51.1, 50.3 (br), 40.7 (br), 40.3 (br, 2 carbons), 33.5 (br), 31.3, 30.6, 29.8, 26.2, 24.0, 22.0, 19.8.

Trifloroacetic acid was added to a solution of this compound in CDCl$_3$, and the ^{1}H NMR was obtained again. **^{1}H NMR** (CDCl$_3$+CF$_3$COOH): δ 7.03 (1H, bs), 3.91 (1H, d, J = 11.0 Hz), 3.70 (1H, m), 3.23 (1H, m), 2.96 (1H, m), 2.65 (1H, m), 2.60-2.45 (2H, m), 2.30 (1H, ddd, J = 10.0, 10.0, 3.0 Hz), 1.00 (3H, d, J = 6.0 Hz).

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INTRODUCTION - The Tenuazonic Acid - *Mycena citricolor* Interaction

The American leaf spot disease of coffee, "ojo de gallo", is one of the most serious diseases of coffee in Latin America, and is caused by the fungus *Mycena citricolor* (Berkeley & Curtis) Saccardo [1,2]. Losses from this disease have been estimated to cause the loss of up to 50% of the annual coffee crop in Costa Rica [3]. The fungus produces necrotic lesions on the leaves, young stems and berries. Light brown circular spots 0.5 to 1.0 cm in diameter form on the leaves, and in the dry season the diseased tissue falls out, leaving a circular hole in the leaf, hence the term 'ojo de gallo' which is Spanish for rooster's eye. The leaves are able to sustain a large number of infections, and remain viable unless one of the necrotic lesions occurs along the principle vein, in which case, the infected leaf will fall. The effect of disease on coffee yield is due mainly to defoliation [4].

In addition to coffee, *Mycena citricolor* also attacks many other species of plants including cacao, citrus and *Cinchona officinalis* L., a medicinal plant which is a source of quinine. Consequently, a number of efforts have been made to understand the biology [1, 4 and references therein] and biochemistry [3,5,6] of *M. citricolor*, and to develop methods to eliminate or control the spread of the disease or reduce the damaging effects of the pathogen [3,7]. It has been determined that oxalic acid produced by *Mycena citricolor* is responsible for the necrosis observed in the American leaf spot disease of coffee [7]. Effects of the disease were mitigated when coffee leaves were sprayed with calcium hydroxide (lime and water) to neutralize the oxalic acid produced by the pathogen [8].

Mycena citricolor

Mycena citricolor is a Basidiomycetes in the order Agaricales [9]. *M. citricolor* has been known by a variety of other names including *Stilbum flavidum* Cke., *Stilbella* *flavida* (Cke.) Kohl, *Agaricus citricolor* Berk. and Curt. and *Omphalia flavida* (Cke.) Maubl. and Rangel [1].

Mycena citricolor produces two types of fruiting bodies: the gemmiferous stage (asexual) and the basidial or perfect stage (sexual). The asexual stage is the main means of propagation, and consists of two parts: the stalk (or the pedicel) and the head (or the gemma). Gemmae are produced abundantly on infected plants. In contrast, the perfect or the sexual stage forms infrequently under natural conditions [1].

The sexual stage of *M. citricolor* is a small yellow mushroom (basidiocarp) about 1.0 to 1.5 cm in height. Basidiocarps of the fungus are 2 to 4 times larger than the stalked gemmae. They are observed rarely under field conditions, however on culture media they have been observed under a variety of conditions. The have been produced on bread-water agar and corn-meal agar [10], on bread water agar and oatmeal agar [11], potato dextrose agar, millet-seed agar and unhusked rice-seed agar [12].

Basidiocarps have also been observed in dual culture, that is, when cultured with other fungus. For example, *Penicillium oxalicum* appearing as a lab contaminant in a culture of *M. citricolor* induced basidiocarp formation in *M. citricolor* near the zone of contact [1]. Other fungi including *Penicillium palitans*, *P. cyclopium*, *P. brevi-compactum*, *P. viridicatum*, *Cladosporium sp.* *Phycomyces blakesleanus*, *Alternaria tenuis* (recently reclassified as *A. alternata* [13]) [1], and *Talaromyces flavus* [14] have also been found to induce basidiocarp formation in *M. citricolor* .

Studies on the stimulating activity of *P. oxalicum* on basidiocarp formation in *M. citricolor* were done in an attempt to identify the nutritional requirements for basidiocarp production by *M. citricolor*, and to determine the effect of *P. oxalicum* culture fluids on *M. citricolor* [1]. The inducing material appears to be a heat stable weak organic acid found in the culture filtrate of *P. oxalicum*. In this study, *P. oxalicum* was grown in liquid culture under a variety of conditions and for varying lengths of time. The basidiocarp inducing activity of each sample was tested by placing a liquid sample from the culture on a Petri dish containing a mycelium of *M. citricolor* on potato dextrose agar. The activity was found only in the culture fluids and not in the mycelium of *P. oxalicum*. Also, the activity was not affected by autoclaving. An important factor in the bioassays was that mushrooms were only produced under diurnal lighting conditions. Cultures of *M. citricolor* growing in continuous darkness or in continuous light fail to produce any mushrooms. Lighting had little or no effect on the production of the stimulating activity in the culture fluids of *P. oxalicum*. The highest basidiocarp stimulating activity was obtained when *P. oxalicum* was grown as a still culture for 1 week in potato dextrose broth.

In one of the more recent studies [15], the stimulating effects of *Talaromyces flavus*, *Alternaria alternata*, and *Penicillium sp.* on basidiocarp formation in *M. citricolor* were compared. It was found that of these species, *A. alternata* was the best inducer (Plate 1).

The goal of the present research was to isolate the compound(s) produced by *A. alternata* (Fries) Keisler responsible for basidiocarp stimulation in *M. citricolor*.

Alternaria alternata

Alternaria alternata is known as an 'imperfect' fungus since it appears to lack (or has yet to be discovered) a sexual phase (perfect stage) of reproduction. Hence, taxonomically, *Alternaria alternata* is found in the sub-division Deuteromycotina, in the form-class Deuteromycetes, and in the form-family Dematiaceae [9]. "*Alternaria* is a large, universally occurring genus. Several form-species are found as saprobes on dead and dying plant parts in the soil from which the conidia [asexual spores] are picked up by the wind, and they invade laboratories where they are troublesome contaminants of cultures. *Alternaria* conidia also occur abundantly in house dust and are one of the chief fungal causes of allergy [16]". Species of *Alternaria* are found in many field and feed crops.

These species of fungi are responsible for many plant diseases including tobacco brown spot, tomato blight, potato early blight, citrus seedling chlorosis and blackspot disease of Japanese pear, to name a few [17]. Grain-crops, hay and silage are often contaminated with this fungi. "Grain which had been previously infected with *A. humicola* and *A. alternata* (Fries) Keisler *(A. tenuis*, Auct. and *A. longipes)* were believed the source of several outbreaks of moldy grain toxicosis in man in the USSR during World War II [17]."

The presence of *Alternaria* toxins in food has stimulated a number of investigations in the areas of isolation and identification of toxins produced by this fungi [19-25], analytical methods for the separation and purification of toxins present [26,27], relationships between structure and phytotoxicity of some of the toxins [28,29], and effects of substrate on metabolite production [30]. As well, some reviews have been written on the structure and toxicity of the *Alternaria* metabolites [13,17,31]. Figure 1 displays some of the important toxic metabolites found in *Alternaria*.

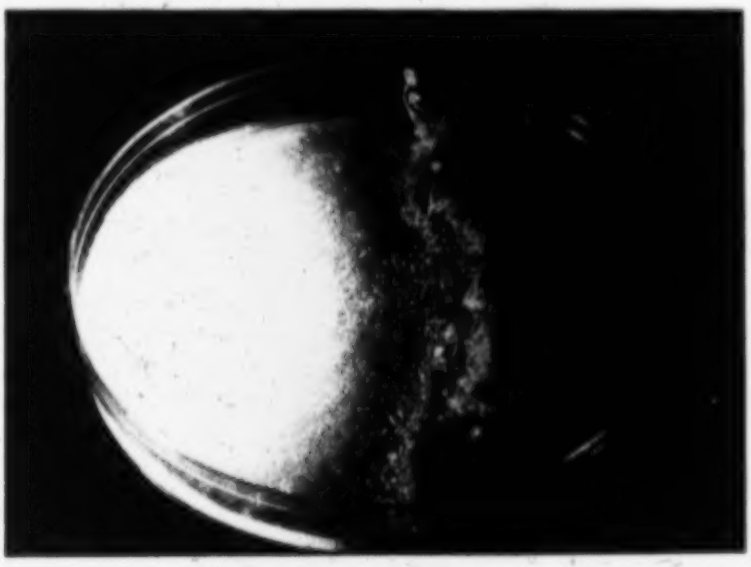
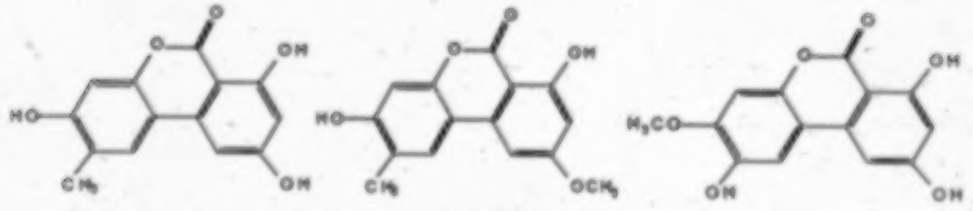
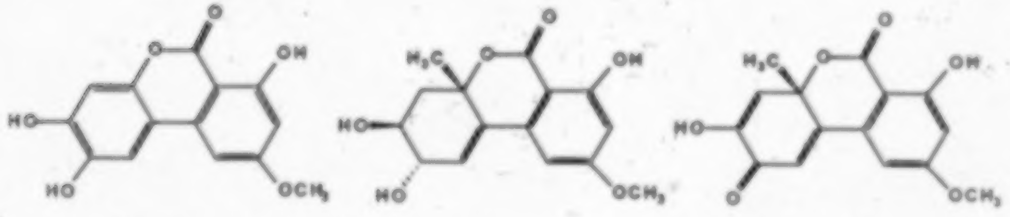


Plate 1: Co-culture of *Mycena citricolor* (isolate UAMH# 6384) with *Alternaria alternata* (isolate UAMH# 6385) showing basidiocarp formation after 3 weeks of growth.

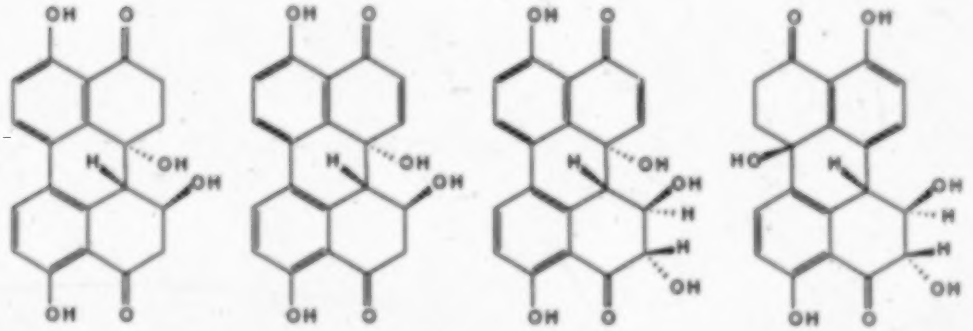
Figure 1: Some toxic metabolites found in the *Alternaria* species.



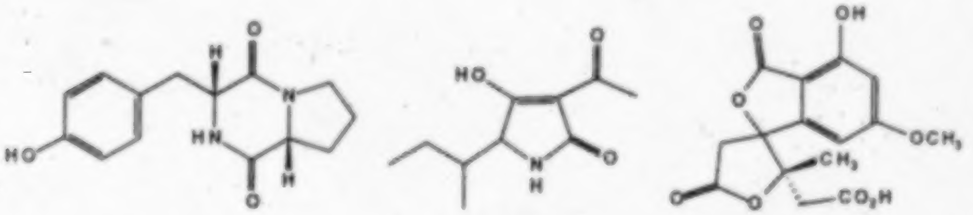
Alternariol [31] Alternariol methyl ether [31] Altenuisol [31]



Altertenuol [17] Altenuene [17] Dehydroaltenusin [17]



Altertoxin I [19] Alteichin [19] Alterlosin I [19] Alterlosin II [19]

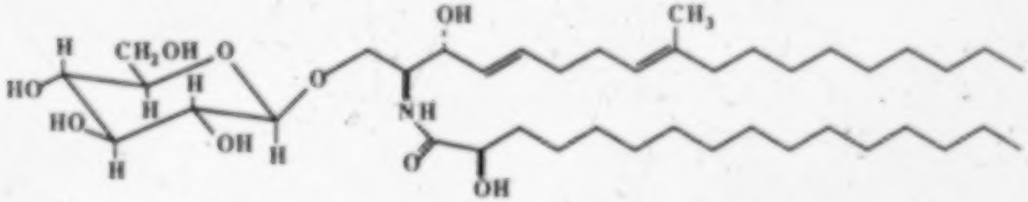


Maculosin [19] Tenuazonic acid [31] Altenuic acid II [17]

RESULTS AND DISCUSSION - *Mycena citricolor*

The term 'probiosis' has been used to describe the stimulation of one micro-organism by another [32], and may be used to describe the effect of *Alternaria alternata* on *Mycena citricolor*. Probiosis may be important in biological control, survival, and reproduction of a micro-organism [32]. The stimulation of fruiting activity in *M. citricolor* by *A. alternata* and other fungi may be a survival mechanism, activated under stress to ensure its continued existence. Induction of fruiting activity has been observed by other fungi in the Basidiomycetes class. A fruiting inducing substance has been isolated from *Cladosporium cladosporioides* [33], and identified as the water soluble and heat stable compound cerebroside [34] (Figure 2) which stimulates fruiting in *Schizophyllum commune*, a fungus in the Basidiomycetes class.

Figure 2: The main active substance (cerebroside) responsible for fruiting in *S. commune*.



Previous work on the stimulation of fruiting activity in *M. citricolor* by *Penicillium oxalicum* showed the active component(s) to be water soluble (activity would not extract into organic solvents), heat stable and found only in the culture filtrates [1]. In previous research in these laboratories, the culture fluids of *A. alternata* were found to stimulate fruiting activity in *M. citricolor*. Furthermore, the active substance was found to be heat stable and water soluble, and the activity was not extractable into organic solvents. However, the active compound was not isolated.

Reconfirming mushroom stimulation and establishing culture conditions.

Attempts were made in the present investigation to confirm the activity of culture fluids of *A. alternata* using samples obtained from the previous investigation in this laboratory. Unfortunately, none of the samples induced fruiting body formation in cultures of *M. citricolor* (strain T-2) at varying concentrations under a variety of conditions. Accordingly, a re-examination of the stimulating effects of live cultures of *A. alternaria* on *M. citricolor* was undertaken.

Alternaria alternata (UAMH 5602) and *M. citricolor* (T-2) were grown in co-culture under various environmental conditions, including growing the cultures in continuous darkness, continuous light, diurnal lighting (12 hours of darkness followed by 12 hours of light), using fluorescent versus incandescent lighting, in cool verses warm temperatures, and using different media. None of these experiments showed any stimulation of mushroom formation in *M. citricolor* by *A. alternata*, although in some cases inhibition zones were observed.

Two new isolates of *M. citricolor* (T-2 re-isolated from a coffee leaf, and strain UAMH 6384 isolated from *Erogoga acuminata*, medicinal plant found in Costa Rica) were obtained as well as a new isolate of *A. alternata* (UAMH 6385). In contrast to the earlier results co-cultures of these isolates showed mushroom stimulation. The greatest mushroom stimulation was observed with *M. citricolor* (UAMH 6384) and *A. alternata* (UAMH 6385) using half strength potato dextrose agar (PDA) media. Furthermore, mushroom stimulation was observed only under alternating light - dark conditions, confirming the need for diurnal lighting for mushroom stimulation [1]. The medicinal plant isolate of *M. citricolor* (UAMH 6384) displayed greater sensitivity to the mushroom stimulant(s) and therefore was used in most of the future bioassays to determine mushroom stimulation activity.

Mycena citricolor degenerates when it is grown on artificial media for long periods of time. The strain T-2, grown for a number of generations on PDA media lost its ability to produce fruiting bodies when stimulated by *A. alternata*. However, after this strain was re-isolated from a coffee leaf, the ability to produce fruiting bodies was re-generated.

Screening of *Alternaria alternata* for production of the active metabolite(s).

After re-establishment of fruiting activity in *M. citricolor*, studies on the production of the stimulating substance in *A. alternata* were performed. Still and shake cultures of *A. alternata* were grown and harvested. The culture fluids were extracted with ethyl acetate, freeze dried and triterated with methanol. These extracts were examined for mushroom stimulation activity using a bioassay called the 'half plate method' (see experimental section). The solutions were autoclaved to ensure sterilization before being tested. Stimulation activity was not found for the ethyl acetate extracts, or in the insoluble residues after trituration with methanol, but activity was found in the methanol soluble extracts of both the shake and still culture freeze dried broths. The appearance of activity in only the methanol soluble portion suggests the active compound is very polar and organic, and its ability to withstand autoclaving indicates that the compound is heat stable.

Since shake cultures were observed to grow much faster than still cultures of *A. alternata*, a time study on the production of the mushroom stimulant in *A. alternata* shake cultures was undertaken. The culture fluids from shake cultures grown for different lengths of time were tested for their ability to stimulate mushroom growth. This experiment showed the stimulant was present in culture fluids obtained between four and ten days of growth, but no stimulant was observed with fluids obtained before or after this time period. Another interesting observation was that the mycelial mass and the pH of the culture fluids reached their maximum value between four and ten days (Table 3, experimental section). It may be possible that after 10 days of growth in shake culture, *A. alternata* had exhausted all available nutrients from the media, and began to degrade its own metabolites, including the mushroom stimulating substance.

Previously, as well as in the present work, it was shown that the mushroom stimulating compound is present in the culture fluids of *A. alternata*. However, the occurrence of this metabolite in the mycelium of this fungus was not previously clearly demonstrated. Accordingly the dried mycelium and the freeze dried broth from a 7-day shake culture of *A. alternata* were separately extracted. Testing of each extract for mushroom stimulation revealed that mushroom stimulating activity was present only in the ethyl acetate and ethanol extracts of the broth. This observation is similar to that reported for *Penicillium oxalicum* where the mushroom stimulation activity was found only in the culture fluids and not in the mycelium [1].

Isolation of the mushroom stimulating compound from *Alternaria alternata*.

The mushroom stimulating compound produced from *A. alternata* was isolated from the methanol soluble portion of a still culture of the fungus. A still culture extract was examined instead of a shake culture since the shake culture bioassays were still in progress at this time, and the activity of this still culture extract had already been confirmed. This extract was subjected to gel filtration chromatography (Sephadex LH20) using methanol as the eluting solvent. Screening of each of the fractions for biological activity showed one of the intermediate fractions induced mushroom formation in cultures of *M. citricolor*. This fraction was then separated using centrifugal counter-current chromatography to give a pure compound which totally inhibited the growth of *M. citricolor* at a concentration of 2 mg per well using the multi-well method (see Plate 2). Subsequently, the compound was tested for biological activity at different concentrations and was found not only to inhibit the growth of *M. citricolor* at high concentrations, but to stimulate mushroom formation at lower concentrations. This compound was identified as tenuazonic acid by spectral analysis and comparison with literature data. Scheme 1 summarizes the isolation of this compound.

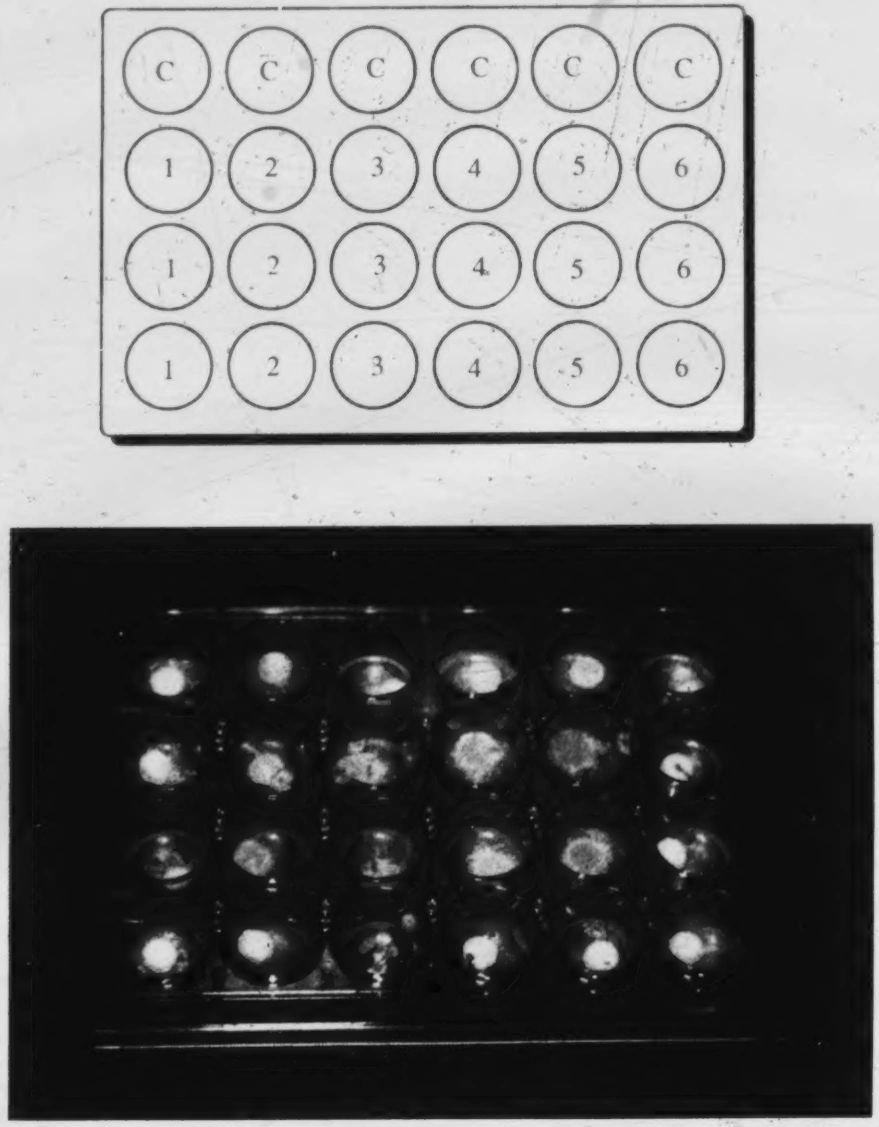
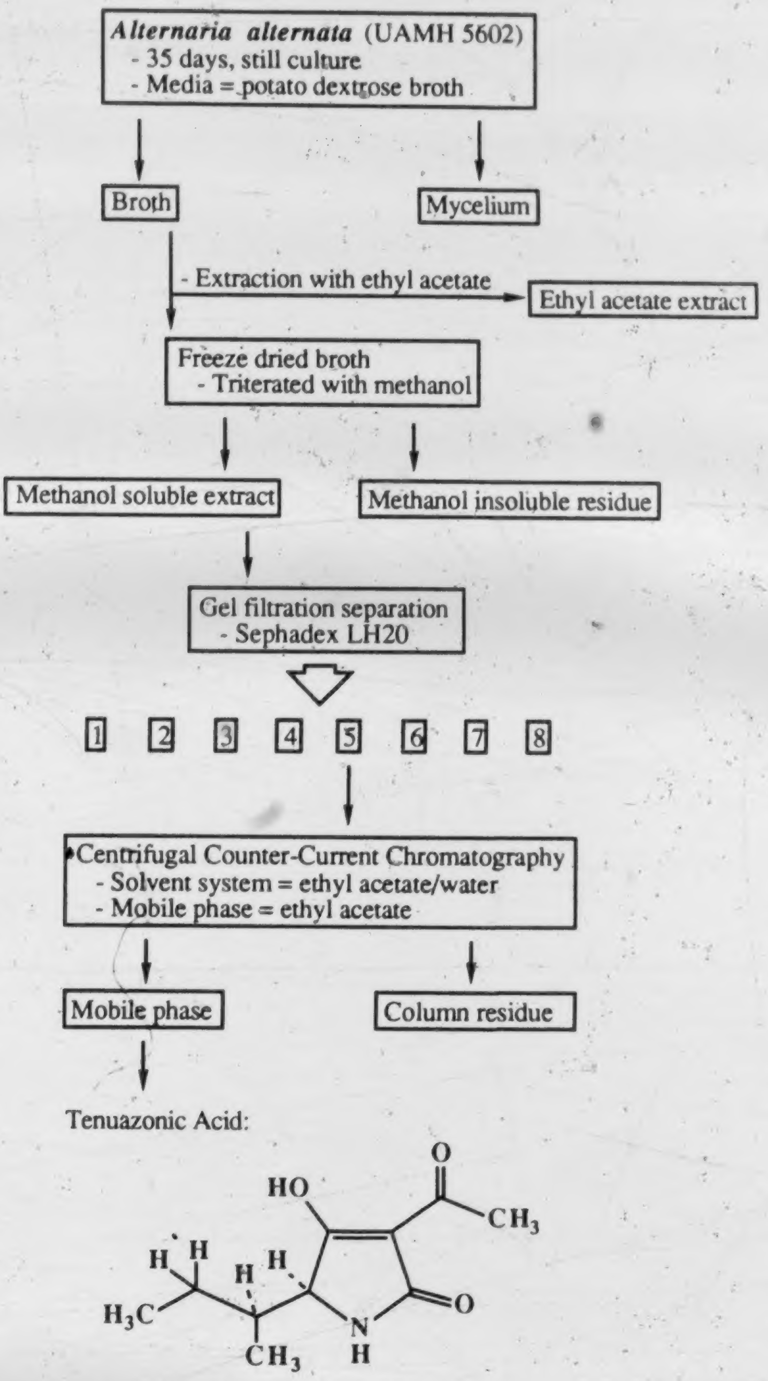


Plate 2: An example of the multi-well method. In this photo, *Mycena citricolor* is being tested against synthetic (+/-) tenuazonic acid (see experimental, Table 13). Using this method, three replicates at each concentration are tested in each row, and one well in each row is left as a control. The multi-well method has the advantages that only small amounts of test material are needed relative to the half-plate method, and results are usually obtained earlier.

Scheme 1: The isolation procedure for the mushroom stimulating compound.


Alternaria alternata (UAMH 5602)
- 35 days, still culture
- Media = potato dextrose broth
Broth
Mycelium
- Extraction with ethyl acetate
Ethyl acetate extract
Freeze dried broth
- Triterated with methanol
Methanol soluble extract
Methanol insoluble residue
Gel filtration separation
- Sephadex LH20
1
2
3
4
5
6
7
8
Centrifugal Counter-Current Chromatography
- Solvent system = ethyl acetate/water
- Mobile phase = ethyl acetate
Mobile phase
Column residue
Tenuazonic Acid:
HO
O
CH3
H
H
H
H
O
H3C
N
H
CH3

Mycena citricolor

When more tenuazonic acid was needed for biological studies, it was obtained using an easier method of isolation[31]. Tenuazonic acid has a pKa of 3.35 [35], and consequently can be extracted from an aqueous solution when the pH is lower than this. Hence, broth extracts of *A. alternata* were acidified to pH 2 with 5% hydrochloric acid and extracted with dichloromethane. The organic extracts were chromatographed over silica gel using dichloromethane - acetic acid as the eluting solvent system to obtain pure tenuazonic acid. The separation of tenuazonic acid using silica gel must be performed quickly since tenuazonic acid appears to decompose slowly on this absorbant.

Characterization of the active metabolite.

The mushroom stimulating metabolite was isolated as a yellow oil which could not be induced to crystallize. The molecular formula was determined from the high resolution mass spectrum as $C_{10}H_{15}NO_3$, and was confirmed by the chemical ionization mass spectrum.

The ^{13}C NMR spectrum showed the presence of 10 different carbon signals, verifying the number of carbon atoms indicated by the molecular formula. As well, the ^{13}C NMR showed three signals to be very deshielded (δ 196.3, 185.8, 176.1) suggesting the presence of three carbonyl carbons, one deshielded resonance at δ 103.4 suggesting the presence of an sp^2 hybridized carbon, and a resonance at δ 67.1 indicating the presence of a carbon atom attached to an electronegative atom such as oxygen or nitrogen.

The ^{1}H NMR spectrum revealed the presence of one methyl group at δ 0.78 (t) coupled to two other hydrogen atoms, one methyl group at δ 1.03 (d) coupled to one other hydrogen atom, and one deshielded methyl group at δ 2.53 (s) coupled to no other hydrogen atoms. Comparison of the spectral data (Table 1) with the literature data for tenuazonic acid confirmed that the active metabolite was tenuazonic acid.

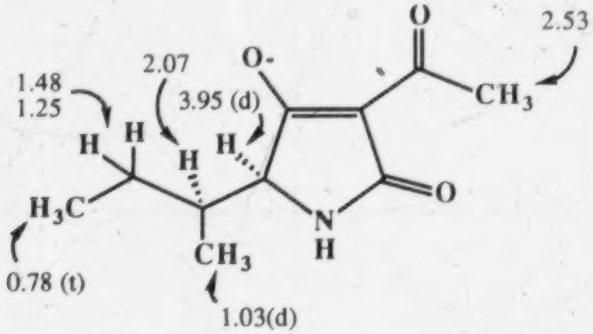
Mycena citricolor

Table 1: Spectral data for tenuazonic acid and the mushroom stimulating metabolite.

Literature data for Tenuazonic Acid [28]		Spectral data for the active metabolite.	
^{1}H NMR (CD_3OD, Na salt) (ppm):		^{1}H NMR (Pyridine-d_5) (ppm):	
0.90	(3H, t, J = 7 Hz, H-7)	0.78	(3H, t, J = 7.5 Hz)
0.98	(3H, d, J = 7 Hz, H-8)	1.03	(3H, d, J = 7.0 Hz)
1.25	(2H, m, H-6)	1.25	(1H, m)
1.90	(1H, m, H-5)	1.48	(1H, m)
2.45	(3H, s, H-10)	2.07	(1H, m)
3.84	(1H, d, J = 3 Hz, H-4)	2.53	(3H, s)
		3.95	(1H, d, J = 3.5 Hz)
^{13}C NMR (D_2O, Na salt) (ppm):		^{13}C NMR (Pyridine-d_5) (ppm):	
12.2	(q, C-7)	12.0	(q)
16.8	(q, C-8)	16.2	(q)
23.1	(t, C-6)	20.8	(q)
28.3	(q, C-10)	24.1	(t)
37.4	(d, C-5)	37.4	(d)
65.7	(d, C-4)	67.1	(d)
104.6	(s, C-2)	103.4	(s)
178.8	(s, C-1)	176.1	(s)
196.2	(s, C-9)	185.8	(s)
199.8	(s, C-3)	196.3	(s)
UV (H_2O, Na salt) λ_{max} (log ε) (nm):		UV (CH_3OH) λ_{max} (log ε) (nm):	
240	(4.06)	240	(3.91)
280	(4.16)	278	(4.05)

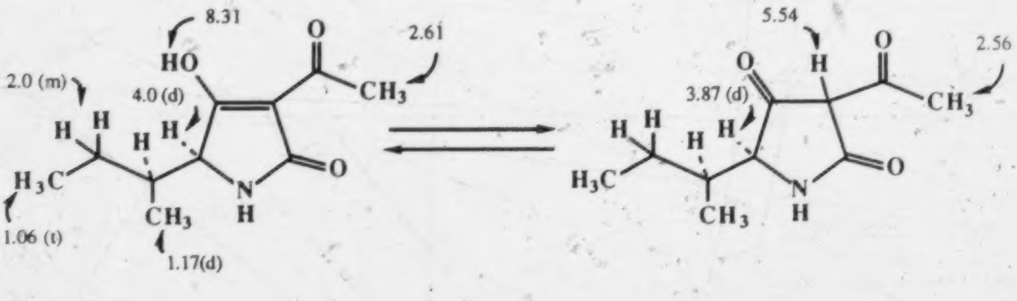
Tenuazonic acid was first isolated in this study as a salt since it showed poor solubility in organic solvents relative to its solubility in water. The counter-ion of this salt was not determined. Because of its poor solubility in chloroform, the salt of tenuazonic acid was dissolved in pyridine-d_5 for NMR analysis. Even when the free acid of tenuazonic is dissolved in pyridine-d_5, it exists as an anion due to the basic nature of pyridine (pKa = 5.2) compared to tenuazonic acid (pKa = 3.35 [35]). Figure 3 displays the ^{1}H NMR spectrum assignments for tenuazonic acid in pyridine-d_5.

Figure 3: ^1H NMR assignment of tenuazonic acid in pyridine-d_5.



Tenuazonic acid is a tetramic acid which can exist as a mixture of two tautomers depending on the pH of the solution. In pyridine, it exists as the delocalized anion (Figure 3). However, two tautomers are observed when the free acid is dissolved in a neutral organic solvent. Thus, tautomerization can be detected in the ^1H NMR and ^{13}C NMR spectra when the compound is dissolved in $CDCl_3$. The assignment of the ^1H NMR signals for the two tautomers of tenuazonic acid are shown in Figure 4 [35].

Figure 4: Tautomers of tenuazonic acid, and their ^1H NMR signals in $CDCl_3$.



Tenuazonic acid is laevorotatory ($[\alpha]_D$= -132* in methanol [35]), however on standing the rotation slowly becomes less negative, and the compound eventually crystallizes. The same observation is made when tenuazonic acid is boiled in aqueous alkaline solution [13,25], and indicates that epimerization of the anomeric center next to the nitrogen atom occurs leading to the partial isomerization of tenuazonic acid to allo-tenuazonic acid.

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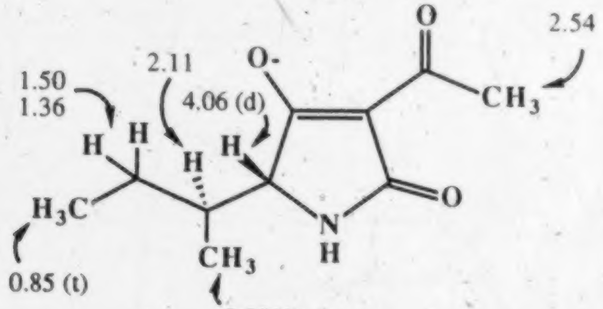
* A range of optical rotations for (-) tenuazonic acid have been observed from -95 to -170 [28].

Allo-tenuazonic acid can be detected by ^{13}C and ^{1}H NMR. However, in deuterochloroform the spectra of the mixture of tenuazonic acid and allo-tenuazonic acid is complicated by the effects of tautomerization of both compounds. Because of this problem and the fact that tautomerization does not occur in pyridine-d_5, pyridine-d_5 was used routinely for NMR studies of these compounds. Figure 5 displays the ^{1}H NMR assignments for allo-tenuazonic acid in pyridine-d_5.

Figure 5: ^{1}H NMR assignment of allo-tenuazonic acid in pyridine-d_5.

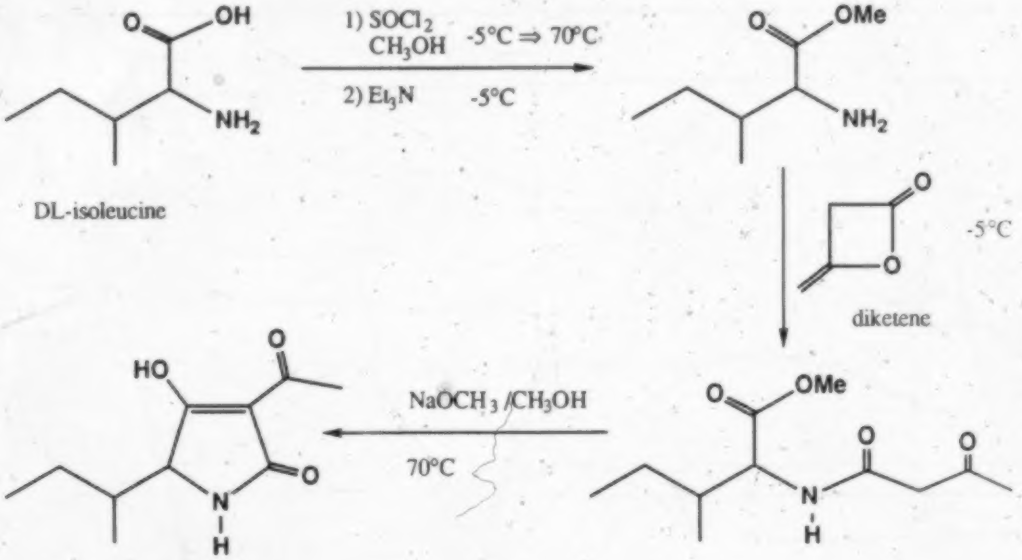


Synthesis of tenuazonic acid.

Since a minor impurity in tenuazonic acid could conceivably have been responsible for the inhibition of growth and/or the stimulation of fruiting activity in *M. citricolor*, tenuazonic acid was synthesized according to a literature procedure [28] for testing of mushroom inducing activity and for growth inhibition of *M. citricolor*. Because of the ready availability of DL - isoleucine, (+/-) tenuanzonic acid was initially synthesized (see

Scheme 2). (The natural form of tenuazonic acid is laevorotatory, and will hereafter be referred to as (-) tenuazonic acid.)

Scheme 2: Synthesis of (+/-) tenuazonic acid.

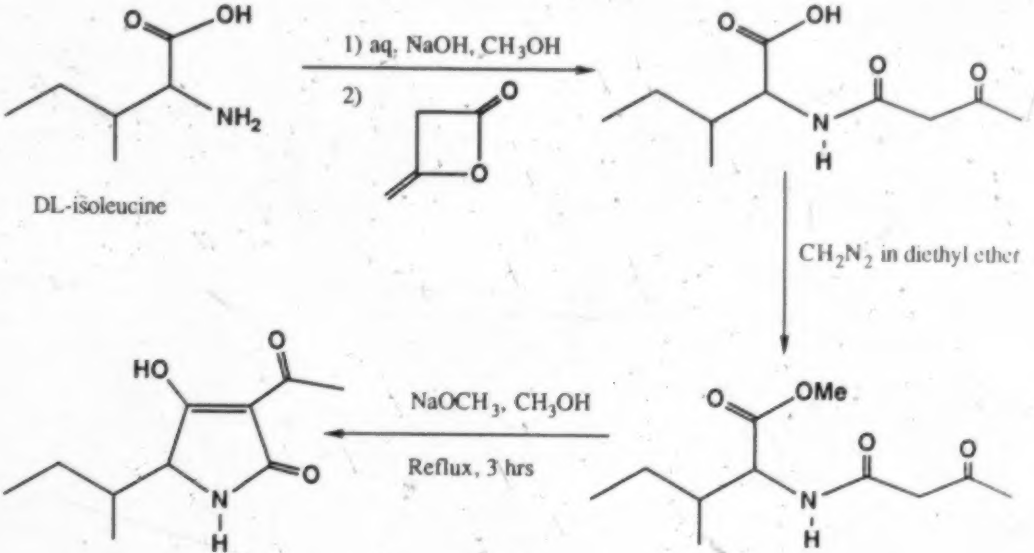


DL-tenuazonic Acid [(+/-) tenuazonic acid]
(41% yield)

In addition, (+) and (-) tenuazonic acid were synthesized according to Scheme 2 from (D) and (L)-isoleucine respecively. Each of the synthetic compounds contained some allo-tenuazonic acid (detected by ^{1}H and ^{13}C NMR, see experimental). Isomerization of tenuazonic acid to allo-tenuazonic acid is possible during the last step of the synthesis where cyclization conditions involve the use of sodium methoxide in methanol.

The method of synthesis of tenuazonic acid outlined in Scheme 2 consistently gave low yields of (-) and (+) tenuazonic acid (9-18% yield), and so an alternate synthetic method was examined [29]. This method was used only to synthesize (+/-) tenuazonic acid. However, it was found to be experimentally easier and resulted in slightly higher yields than any of the previous preparations of tenuazonic acid. Scheme 3 outlines the steps for this synthetic method.

Scheme 3: Improved method for the synthesis of tenuazonic acid.



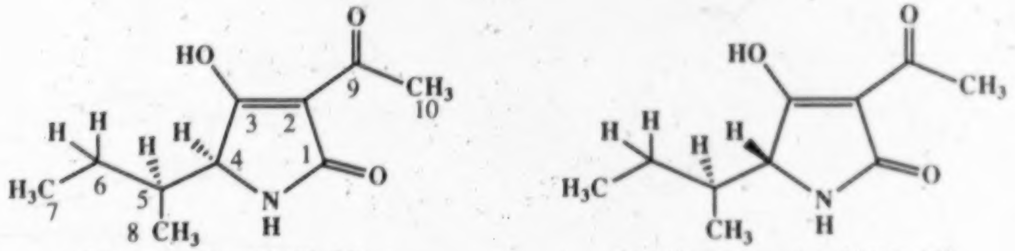
DL-isoleucine

DL-tenuazonic Acid [(+/-) tenuazonic acid]
(43% yield)

In total, four different stereo-isomers of tenuazonic acid were synthesized (Figure 6). The optical isomers of tenuazonic acid were always contaminated with allo-tenuazonic acid. From examination of the relative integration of the signals in the ^{1}H NMR spectra, the amount of allo-tenuazonic acid present in relation to tenuazonic acid ranged from 30 to 50% of the mixture.

Mycena citricolor

Figure 6: Isomers of tenuazonic acid [28,35].

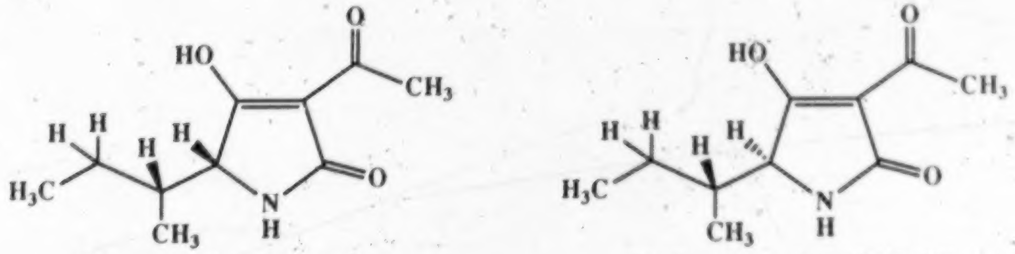


(L)-tenuazonic acid (4S, 5S)

$[\alpha]_D$=-132°

(D)-allo-tenuazonic acid (4R, 5S)

$[\alpha]_D$=+176°



(D)-tenuazonic acid (4R, 5R)

$[\alpha]_D$=+132°

(L)-allo-tenuazonic acid (4S, 5R)

$[\alpha]_D$=-176°

Biological testing of Tenuazonic Acid.

The first bioassays on synthetic tenuazonic acid were performed using (+/-) tenuazonic acid. Positive results for mushroom stimulation and growth inhibition of *M. citricolor* using synthetic (+/-) tenuazonic acid would help to confirm that mushroom stimulation of *M. citricolor* was by natural tenuazonic acid, and not by a minor impurity. The synthetic compound was tested for biological activity using two different methods (multi-well and half-plate method). Both methods showed this compound to be an inhibitor of mycelial growth, but neither of the methods confirmed mushroom stimulation activity.

In initial work to confirm the mushroom stimulation activity of *A. alternata* on *M. citricolor,* environmental conditions were found to play a very important role in the production of fruiting bodies. The negative results obtained on the stimulating effects of

(+/-) tenuazonic acid on fruiting activity could have been due to inadequate control of environmental conditions in the laboratory. In order to confirm this, a study was undertaken where bioassays were performed on natural (-) tenuazonic acid and synthetic (+/-) tenuazonic acid at the same time and under the same growing conditions. The half plate method was used in order to measure and compare the inhibitory effects of both compounds along with the stimulating activity. Inhibition of mycelial growth was quantified by comparing the diameter of fungal growth on the test side of the Petri dish with that on the control side. Mushroom stimulation was quantified by taking the average number of mushrooms on the test plates for each different concentration.

This study showed natural (-) and synthetic (+/-) tenuazonic acid were both able to stimulate mushroom production and inhibit growth of *M. citricolor*, although (+/-) tenuazonic acid was less effective than (-) tenuazonic acid (Figure 7). In all of the different bioassays performed for this experiment, at no time were mushrooms observed on the control side of the plate (see Plates 3-6). Therefore, the appearance of mushrooms was due only to the presence of the test solutions, and not to some outside influence. This strongly suggests that tenuazonic acid is a causal agent in the stimulation of fruiting activity and growth inhibition of *M. citricolor*, and that a minor impurity in the natural compound is not responsible for this activity.

The following photos show the effects different concentrations of natural (-) tenuazonic acid on the growth and stimulation of *Mycena citricolor*.

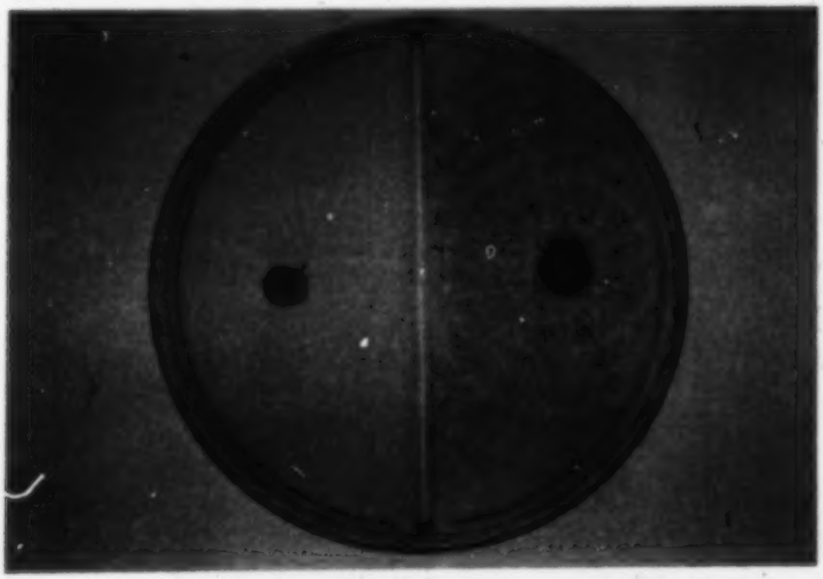


Plate 3: *Mycena citricolor* with natural (-)tenuazonic acid [4.90 x 10^{-3}M].

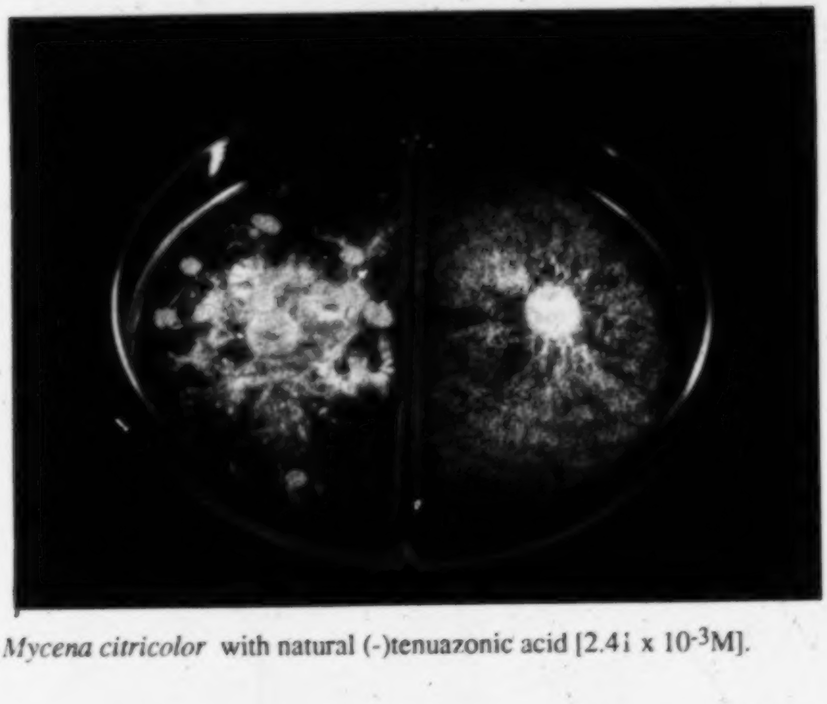


Plate 4: *Mycena citricolor* with natural (-)tenuazonic acid [2.41 x 10^{-3}M].

Mycena citricolor

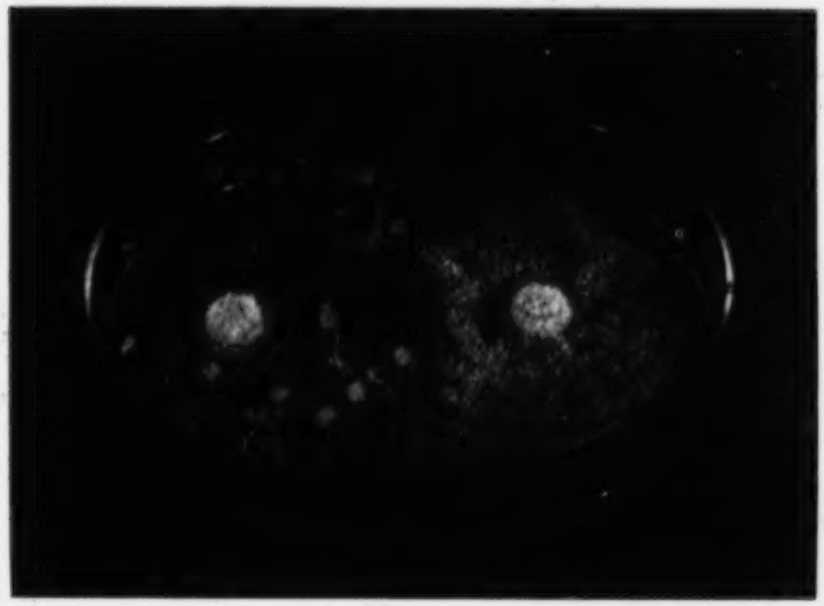


Plate 5: *Mycena citricolor* with natural (-)tenuazonic acid [1.21×10^{-3}M].

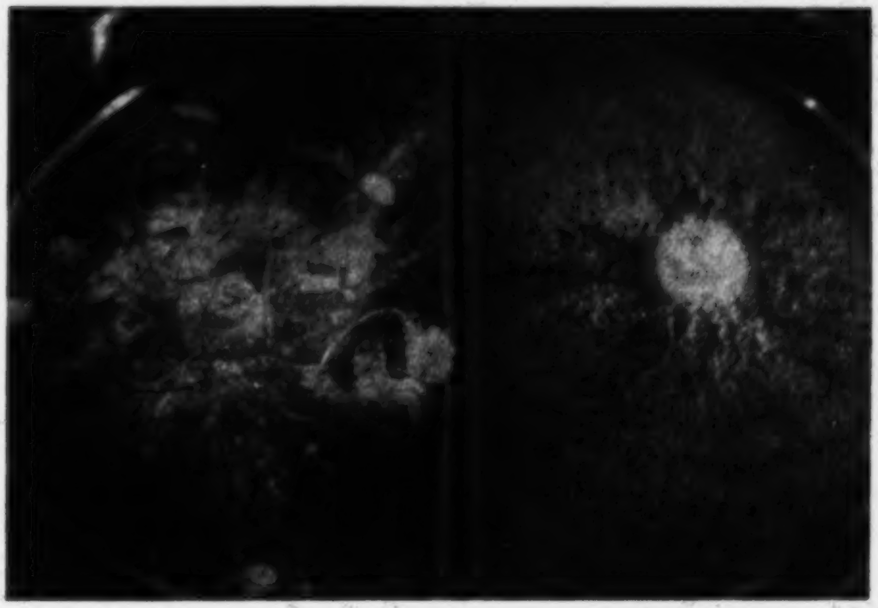
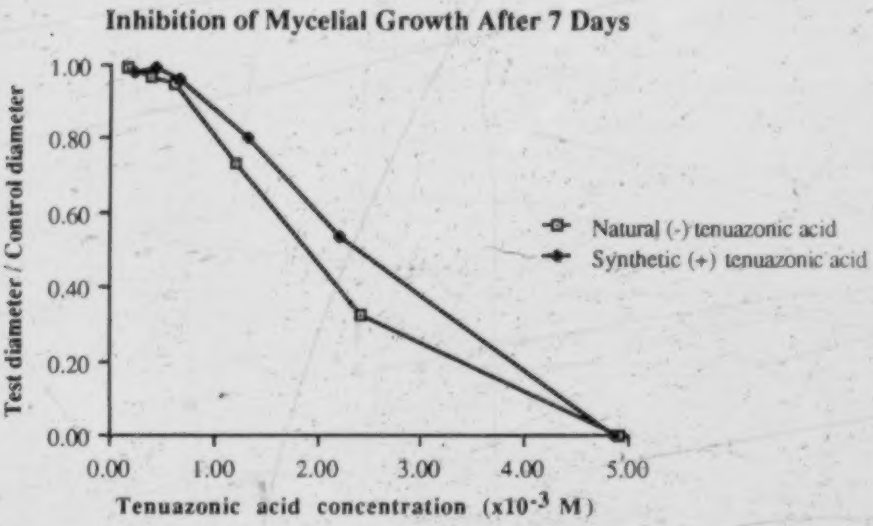
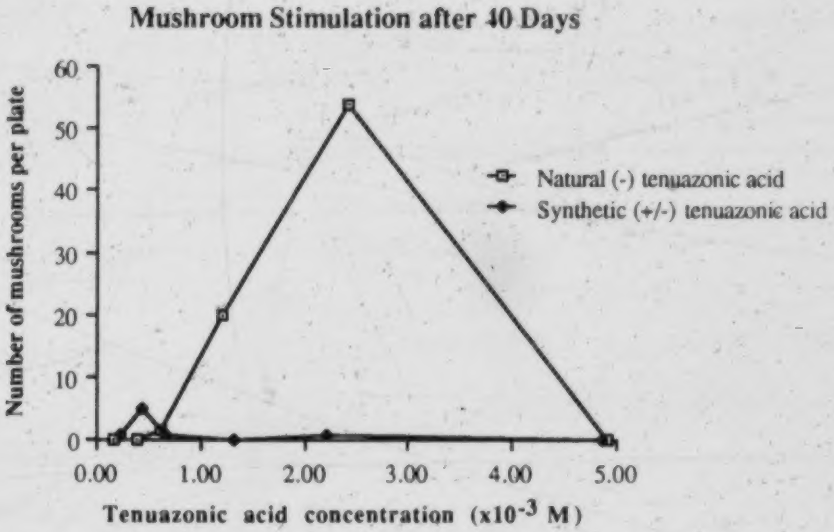


Plate 6: *Mycena citricolor* with natural (-)tenuazonic acid (Closeup of plate 4).

Figure 7: A comparison of the effects of (-) and (+/-) tenuazonic acid on *M. citricolor*.





The results obtained from the comparison of (-) and (+/-) tenuazonic acid showed a relatively close comparison in the inhibition activity, but a large difference in the mushroom stimulation activity. Previous studies by others on the toxic effects of tenuazonic acid on rice roots found (-) tenuazonic acid to be more active than (+) tenuazonic acid on the inhibition of rice root growth, and modifications to its structure only decreased the activity [28].

Accordingly; biological testing was performed using synthetic samples of (+), (-), and (+/-) tenuazonic acid. The the half plate method was used in this study (see Plates 7 and 8). In previous bioassays, cultures were grown in a large room where the temperature often fluctuated by 5 to 10°C, and where the control of lighting conditions was poor. In this experiment, bioassays were placed in a growth chamber where the temperature was a constant 23°C, and the lighting was set to a 12 hour photo-period. The inhibition activity of (+), (-) and (+/-) tenuazonic acid was measured by comparing the diameter of the mycelial growth on the test side of the plate with that that on the control side for each solution 8 days after inoculation. The amount of mushroom stimulation activity was quantified by counting the number of mushrooms observed for each solution 29 days after inoculation. The results (Figure 8) show that (-) tenuazonic acid is the most active in mushroom stimulation and growth inhibition of *M. citricolor*, followed successively by (+/-) tenuazonic acid, then (+) tenuazonic acid.

It is interesting to notice that (+) tenuazonic acid is relatively active as an inhibitor and mushroom stimulator of *M. citricolor*. (+) Tenuazonic acid was synthesized from comercial D-isoleucine which contained approximately 10% of the allo isomer. Furthermore, synthetic (+) tenuazonic acid contained approximately 25% allo-tenuazonic acid as determined by ^{1}H NMR analysis. Since (+) tenuazonic acid was not isomerically pure, the activity of this isomer on *M. citricolor* is not well defined.

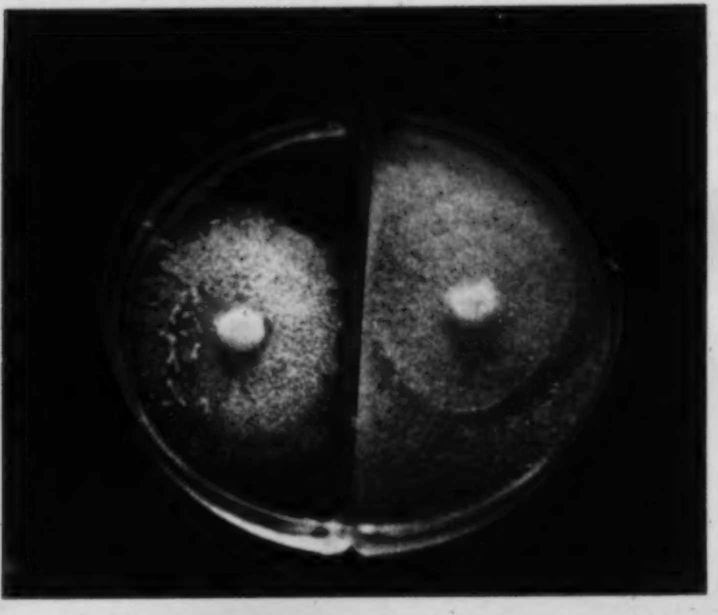


Plate 7: *Mycena citricolor* with synthetic (-)tenuazonic acid [1.69 x 10^{-3}M]

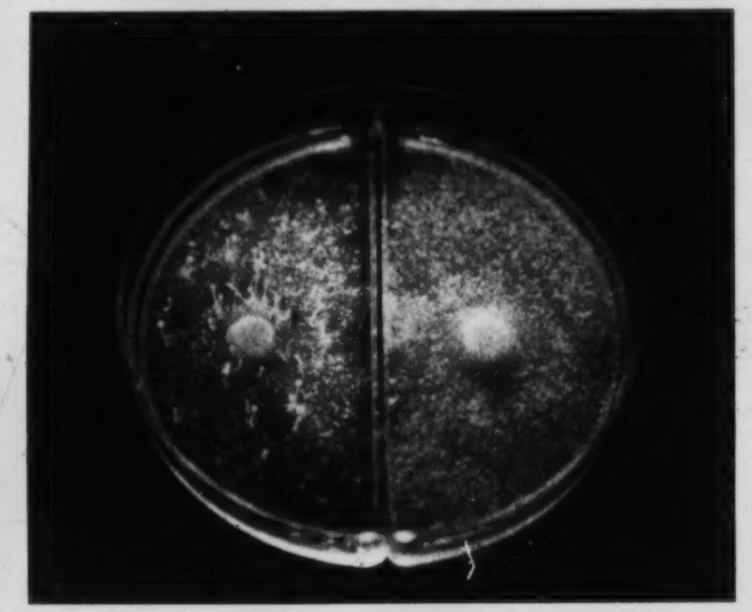
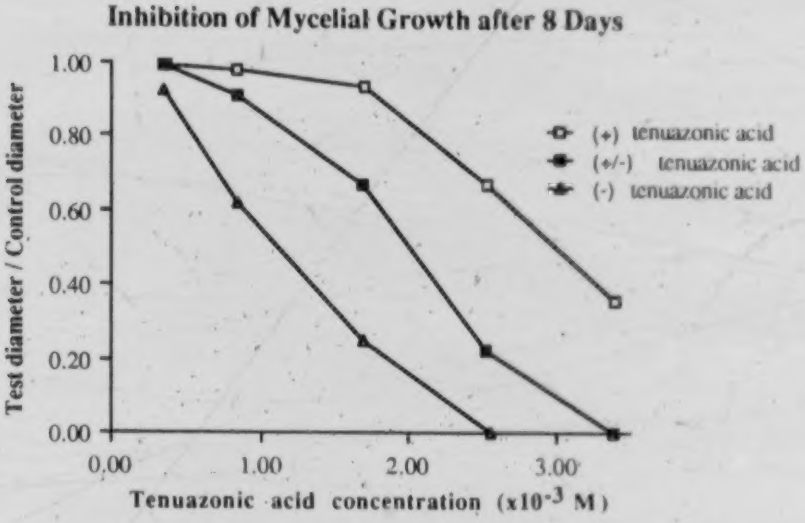
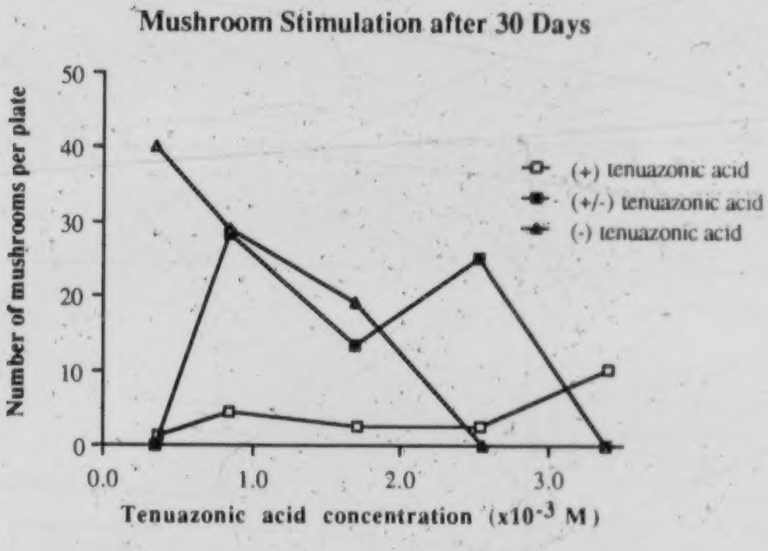


Plate 8: *Mycena citricolor* with synthetic (-)tenuazonic acid [0.85 x 10^{-3}M].

Inhibition of growth is more appearent in these photos than in the previous ones. These were taken approximately 2 weeks after inoculation and show immature mushrooms.

Figure 8: A comparison of the effects of synthetic (+), (+/-), and (-) tenuazonic acid on *M. citricolor*.





Comparison of Figure 7 with Figure 8 indicates that the rate of inhibition for natural (-) tenuazonic acid is quite comparable with that for synthetic (-) tenuazonic acid, and the inhibition rates for (+/-) tenuazonic acid in both experiments are also similar. However, the mushroom stimulation activity appears to be quite different between experiments. Further comparisons of mushroom stimulation activity show that (+/-) tenuazonic acid in Figure 7 has a much lower stimulation activity than (+/-) tenuazonic acid in Figure 8, although one would expect them to be similar. This variability in mushroom stimulation of *M. citricolor* demonstrates the sensitivity of fruiting activity of this fungus to environmental conditions, an observation made earlier in this research when many problems were encountered in attempts to confirm fruiting ability in this fungus. It is apparent that the inhibition of mycelial growth of *M. citricolor* is much less affected by slight changes in environmental conditions.

Most of the previous bioassays were performed using *M. citricolor* (UAMH 6384) isolated from *Erogoga acuminata*, a medicinal plant. Since we were interested originally in the strains of *M. citricolor* isolated from coffee plants, four coffee isolates of *M. citricolor* (MCH2, MCH4, MCH9a, MCH12) were tested for mushroom stimulation by synthetic (-) tenuazonic acid. The multi-well method was used for the bioassays. In previous studies where these strains were tested for basidiocarp-induction response due to *A. alternata*, strain MCH12 formed fertile mushrooms, strains MCH2 and MCH4 formed sterile mushrooms, and strain MCH9a did not form any mushrooms [36]. In this experiment using synthetic (-) teuanzonic acid for stimulation, 20 days after inoculation strains MCH2 and MCH12 showed full development of mushrooms, strain MCH4 showed the development of mushroom primordia, and strain MCH9a showed no mushroom stimulation. All strains showed growth inhibition due to synthetic (-) tenuazonic acid. It is interesting to notice that both studies showed that strain MCH9a is unable to produce fruiting bodies when stimulated.

Tenuazonic acid stimulates various strains of *M. citricolor*. This compound is found in a number of *Alternaria* species including *A. alternata*, *A. citri*, *A. kikuchiana*, *A. mali*, and *A. tenuissima*, which are a widely distributed species of fungi, as well as in other species of fungi including *Phoma sorghina*, *Pyricularia oryzae*, and isolates of *Aspergillus*, and other taxa in Spaeropsidales [13]. Because of its wide distribution, tenuazonic acid may also be responsible for the stimulation of fruiting activity in other species of fungi besides *Mycena citricolor*. The effects of tenuazonic acid on other species of fungi is now being investigated.

Tenuazonic acid is responsible for the inhibition of growth of *M. citricolor*. This observation suggests that tenuazonic acid could be used as a fungicide in the control and spread of American leaf spot disease in coffee or other diseases caused by *M. citricolor*. At concentrations of approximately 2.5×10^{-3} M (0.5 mg/ml) or higher, (-) tenuazonic acid totally inhibits the growth of *M. citricolor*. However, the toxicity of tenuazonic acid has been studied extensively, and it has been found that tenuazonic acid has quite a broad toxicity spectrum. Tenuazonic acid is a growth inhibitor of tumor cells, has viricide, bactericide and insecticide activities, and is toxic to mammals, birds, and plants [13, 28, 37]. Because of the broad range of toxicity of this compound, it is probably unwise to use it as a fungicide on *M. citricolor*.

Preliminary investigation of some other mushroom inducing fungi.

Other species of fungi besides *Alternaria alternata* have been reported to stimulate fruiting activity in *M. citricolor*. The strains of *M. citricolor* available in this lab were tested against two of these other species, *Talaromyces flavus* and *Penicillium oxalicum*, using co-culture methods. The results showed no stimulation of the coffee isolates of *M. citricolor*, however, the more sensitive medicinal plant isolate did show mushroom stimulation by both *T. flavus* and *P. oxalicum*, although this stimulation was poor and variable between replicates. Also, a large inhibition zone was observed in co-cultures of

Mycena citricolor

P. oxalicum with all of the strains of *M. citricolor*, (see Plate 9) but no zone was observed in co-cultures of *T. flavus* with any of these strains (see Plate 10). These results confirm that *P. oxalicum* and *T. flavus* can stimulate fruiting activity in *M. citricolor*. The question as to whether the compounds responsible for this activity in these two fungi are similar to tenuazonic acid, and whether the compound in *P. oxalicum* which is responsible for the large inhibition zone observed on the co-cultures is also responsible for mushroom stimulation remain to be answered.

The large zone of inhibition observed between *M. citricolor* and *P. oxalicum* suggests the possibility of using *P. oxalicum* as a biological control on *M. citricolor*. By treating seeds of corn, peas, soybeans and garbonzo beans with spores of *P. oxalicum*, these seeds were protected against decay by *Pythium* spp., *Rhyzoctonia solani*, and other soil pathogens [38]. *Penicillium oxalicum* may also be useful in the control of *M. citricolor* on coffee.

Previous studies indicated that the mushroom stimulating substance in *P. oxalicum* was water soluble, heat stable, and possibly a weak organic acid [1]. Since tenuazonic acid also has these characteristics, the possibility that the active compounds in *P. oxalicum* and *T. flavus* are similar or identical to this compound was entertained. Consequently, shake and still cultures of *T. flavus* and *P. oxalicum* were grown and harvested. The culture fluids were concentrated, and a portion of each concentrate was basified to pH 9, extracted with dichloromethane, acidified to pH 2, and extracted with ethyl acetate. The ethyl acetate extracts were analyzed by TLC for the presence of tenuazonic acid. Tenuazonic acid forms a very obvious brown-orange spot on silica gel TLC plates when dipped into a solution of phosphomolybdic acid developing solution. However, tenuazonic acid was not detected in any of these extracts. Therefore, the compounds responsible for mushroom stimulation in *T. flavus* and *P. oxalicum* are most likely not tenuazonic acid.

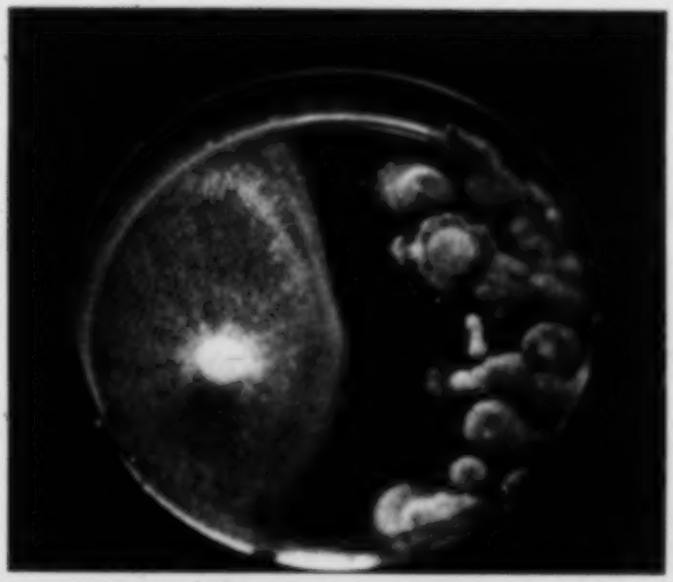


Plate 9: *Mycena citricolor* on co-culture with *Penicillium oxalicum*.

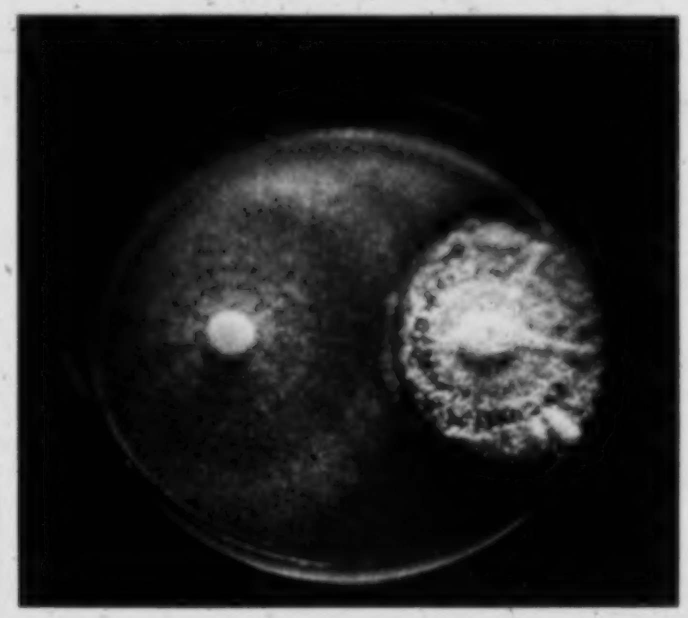


Plate 10: *Mycena citricolor* on co-culture with *Talaromyces flavus*.

These photos show the effect of *Taleromyces flavus* and *Penicillium oxalicum* on *M. citricolor*. Note that in these photos, there is no mushroom stimulation observed.

In an attempt to characterize the active substances in *T. flavus* and *P. oxalicum*, all the broth extracts were screened for mushroom stimulation and for growth inhibition of *M. citricolor*. The results (summarized in Table 18, experimental section) show that the concentrated culture filtrates for the shake and still cultures of both species of fungi inhibited mycelial growth, however inhibition was not apparent in any of the acidic fractions from these concentrates. Mushroom stimulation was apparent only in the culture filtrate of the still culture of *P. oxalicum* and the acidic fraction from this filtrate. Here, mushroom primordia were observed but they never fully developed into mushrooms. In previous studies where shake and still cultures of *P. oxalicum* were tested for mushroom stimulation, only the still cultures were active [1]. The current results are in agreement with these studies, and suggest that any future work on the identification of the active compound in *P. oxalicum* should start with examination of the still cultures of this species.

Conclusion.

In the present study, it has been shown that the metabolite responsible for mushroom stimulation and growth inhibition of *Mycena citricolor* by *Alternaria alternata* is the known compound tenuazonic acid. This activity was confirmed with tenuazonic acid obtained by synthesis. Furthermore, it was determined that the natural isomer of this compound was the most active in mushroom stimulation as well as growth inhibition. One isolate of *M. citricolor* is not stimulated to produce mushrooms by tenuazonic acid, although its growth is still inhibited by this compound. The results from a preliminary examination of *Talaromyces flavus* and *Penicillium oxalicum* suggest that other compounds may be responsible for mushroom induction and growth inhibition of *M. citricolor* since tenuazonic acid was not detected in extracts from cultures of either of these fungi. Furthermore, a metabolite responsible for mushroom stimulation in *P. oxalicum* may be an acidic organic compound as indicated by earlier research [1].

Mycena citricolor

EXPERIMENTAL - *Mycena citricolor*

Source of the Fungi.

A number of strains of *Mycena citricolor* (MCH2, MCH4, MCH9a, MCH12, T-2) were obtained from the Plant Science Department of the University of Alberta. These strains were isolated from diseased coffee leaves collected from the principle coffee growing areas of Costa Rica by Amy Wang. The strain T-2 was previously deposited at the Agriculture Canada Biosystemetics Research Institute and given the accession number DAOM 191786. Another strain of *M. citricolor* was obtained from the Plant Science Department. It was previously isolated in Costa Rica by Dr. J.P. Tewari from a leaf lesion on *Erogoga acuminata*, and isolated onto artificial media in Edmonton by S. Brezden. This strain was deposited at the University of Alberta Microfungus Herbarium, and given the accession number UAMH 6384. *Erogoga acuminata* is a medicinal plant whose common Spanish name is 'epicauhuana'.

One isolate of *Alternaria alternata* (UAMH 5602) from Costa Rica was obtained from the University of Alberta Microfungus Herbarium. Another isolate of *A. alternata* was obtained from the Plant Science Department of the University of Alberta. This strain, previously isolated as an air contaminant in Edmonton by S. Brezden, was deposited at UAMH as UAMH 6385.

Penicillium oxalicum (UAMH 5148) and *Talaromyces flavus* (UAMH 4890) were obtained from the University of Alberta Microfungus Herbarium.

Inoculum Preparation.

The fungal cultures were maintained on slants (media - Potato Dextrose Agar, PDA) in the refrigerator at 4°C. When cultures were needed, a small piece of mycelium and agar was removed from the slant culture and used to inoculate an agar plate (PDA). The plate culture was allowed to grow until the mycelium covered approximately 2/3 of the surface area of the plate (usually 1-2 weeks). Small plugs of mycelium on agar (9 mm) were taken from these plates for bioassays. When liquid cultures were needed, an entire plate with mycelium and agar was emptied into a flask containing 250 ml of sterilized water and blended for a few minutes. Aliquots (20 ml) of this suspension were used to inoculate still or shake cultures.

Media Preparation.

For liquid cultures the organisms were grown on Potato Dextrose Broth (PDB) media at full or half strength. Full strength media was prepared by rehydrating 24 grams of Difco brand Bacto- potato dextrose broth dehydrated media (Formula: ingredients/liter: infusion from potatoes, 200 grams, Bacto - dextrose, 20 grams) in 1 liter of distilled water. Half strength media was prepared by rehydrating 12 grams of PDB in 1 liter of distilled water. The solutions were autoclaved at 121°C for 20 minutes prior to use.

Cultures on solid media were grown on full or half strength Potato Dextrose Agar (PDA) media. Full strength media was prepared by rehydrating 39 grams of Difco brand Bacto- potato dextrose agar dehydrated media (Formula: ingredients/liter: infusion from potatoes, 200 grams; Bacto - dextrose, 20 grams; Bacto - agar, 15 grams) in 1 liter of distilled water. Half strength media was prepared by rehydrating 15 grams of dehydrated PDA in 1 liter of distilled water and amending with 7.5 g/L agar (BDH Agar coarse powder). The media was autoclaved at 121°C for 20 minutes prior to being poured into the sterilized containers.

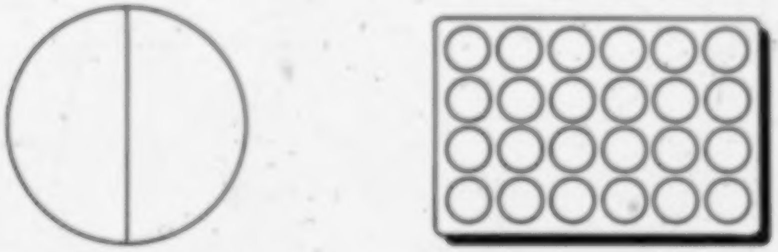
Culture Conditions.

Cultures were grown on still and shake cultures using full and half strength PDB media. Still cultures were grown in Fernbach flasks containing 1L of media, and shake cultures were grown in 1L Erlenmyer flasks containing 500 ml of media.

Bioassay Methods.

In order to test solutions and compounds for biological activity, two types of bioassays were performed. The first one will be referred to as the 'half plate method' and uses 9 cm Petri dishes divided in half by a plastic barrier. The solution to be tested is mixed with full or half strength potato dextrose agar (PDA), autoclaved and poured onto one side of the divided dish. The other side of the dish is prepared with just PDA. When the agar solutions have solidified, both sides of the dish are inoculated with an agar plug (9 mm) containing *M. citricolor*. The dishes are examined for basidiocarp formation and inhibition of growth after three to four weeks of growth. Each solution is tested in triplicate.

Figure 9: Bioassay methods.



Half-Plate Method **Multi-Well Method**

The second bioassay method will be referred to as the 'multi-well method' and uses dishes with 24 individual wells. Into each well is pipetted 1 ml of full or half strength PDA. When the agar has solidified, 0.10-0.20 ml of the sterilized aqueous test solutions are pipetted into each of 3 wells. The fourth well in a row is used as a control. A plug of agar containing *M. citricolor* is then placed in each of the wells, and the growth is monitored for inhibition and basidiocarp formation for a number of weeks. Up to 6 solutions are tested on a single dish.

Confirmation of mushroom stimulation.

To confirm observations by others that *Alternaria alternata* induces mushroom stimulation in *Mycena citricolor* when grown in co-culture [1,15], *A. alternata* (UAMH 5602) and *M. citricolor* (T-2) were co-inoculated on Petri dishes containing PDA (full strength) media. The bioassays were performed under a number of growing conditions including darkness versus light, diurnal lighting (12 hours of light followed by 12 hours of darkness) versus continuous lighting, fluorescent versus incandescent lighting, as well as cool versus warm temperatures. None of the experiments showed basidiocarp stimulation in *M. citricolor* along the colony border with *A. alternata*, although sometimes a zone of inhibition was observed between the two growing fungi.

Two new isolates of *M. citricolor* (UAMH 6384 and T-2, re-isolated from a coffee leaf) and a new isolate of *A. alternata* (UAMH 6385) were obtained from the Plant Science Department of the University of Alberta. The two new isolates of *M. citricolor* were tested for basidiocarp induction using the previous isolates of *A. alternata*. Stimulation was observed for both new isolates of *M. citricolor*, however the medicinal plant isolate (6384) showed much better stimulation than the re-isolated coffee leaf isolate (T-2). Subsequently, Shirley Brezden of the Plant Science Department discovered that the greatest stimulation was observed when the medicinal plant isolate was grown with the new isolate of *A. alternata* using half strength PDA media .

Initial screening and extraction of *Alternaria alternata* metabolites.

Alternaria alternata (UAMH 5602) was grown on still and shake culture using PDB. The still culture (10 L) was harvested after 35 days, and the shake culture (6 L) was harvested after 7 days. The cultures were filtered through cheesecloth to separate the mycelium from the broth. The mycelium was air dried in the fume hood for a few days and set aside. The broth was concentrated to 1/10 volume, extracted with ethyl acetate and freeze-dried. The freeze-dried powder from the broth was triturated with methanol and separated into methanol soluble and triturated insoluble material (see Table 2).

Table 2: Extract yields from culture fluids (10 L) of *Alternaria alternata.*

Source of extract		Mass of extract (g)
Shake culture broth	methanol soluble	60.0
	methanol insoluble	12.0
	ethyl acetate extract	0.0
Still culture broth	methanol soluble	15.0
	methanol insoluble	8.2
	ethyl acetate extract	0.0

Each of these extracts were screened for biological activity using the half plate method. The extracts were each dissolved in 60 ml of water and 2.3 grams of dehydrated PDA powder was added. After autoclaving for 20 minutes at 121°C, each solution was poured onto one side of three divided plates. To the other side of the plates, autoclaved full strength PDA (2.3 g in 60 ml water) was added. When the agar had solidified, the plates were inoculated with *M. citricolor* (UAMH 6384), and grown under diurnal lighting conditions (12 hour photo period). The following table displays these results after 30 days of growth.

Table 3: Biological screening of extracts from *A. alternata* (UAMH 5602).

Culture	Extract from Broth	Concentration (mg/ml)	Observations
Still	ethyl acetate extract	2.0	- no activity
	methanol soluble	12.5	- total inhibition of growth
	methanol insoluble	3.7	- no activity
Shake	ethyl acetate extract	2.5	- no activity
	methanol soluble	17.8	- mushroom stimulation
	methanol insoluble	3.5	- no activity

Time study on the occurrence of the mushroom stimulant(s) in *A. alternata.*

Alternaria alternata (UAMH 6385) was grown on shake culture for varying lengths of time. Ten flasks containing 500 ml of half strength PDB were inoculated with *A. alternata* and placed on the shaker. The broth extracts were bioassayed using the half plate method. One solution was taken off the shaker every second day for 21 days and the broth was filtered from the mycelium and concentrated to 60 ml. Full strength PDA media was added to each of the solutions, and the solutions were autoclaved. The solutions were poured onto the divided plates, and the plates were inoculated using *M. citricolor* (UAMH 6384). The bioassays were monitored for 30 days. A test was considered positive when at least one mushroom was observed in each of the three replicates of a test solution. The following table shows these results.

Table 4: Time study of *M. citricolor* mushroom stimulation by *A. alternata.*

Days on Shaker	*Mycelial Mass (mg)	pH of Broth Extract	Mushroom Activity
2	446	5.6	-
4	2325	8.8	+
6	2668	8.6	+
8	2887	8.7	+
10	2335	8.8	+
12	2084	8.9	-
14	2150	8.9	-
16	2355	8.9	-
18	2080	8.9	-
21	2145	8.9	-

*Dry weight

Screening of shake culture metabolites.

Alternaria alternata (UAMH 6385) was grown on shake culture for 7 days (10 L, PDB). The broth was filtered through cheesecloth to separate the mycelium. The mycelium was air dried, and the broth was concentrated to 1/10 volume and freeze dried.

The dried mycelium and the freeze dried broth powder were extracted using a percolator type extractor. Each extract was successively extracted for 24 hours with dichloromethane (CH_2Cl_2),ethyl acetate (EtOAc), and with ethanol (EtOH). The extracted mycelium was soaked in 20% (v/v) acetic acid for 3 days, air dried and extracted for 3 hours with CH_2Cl_2 and then for 24 hours with EtOH. Each extract was tested for biological activity against *M. citricolor* (UAMH 6384) over 30 days using the multi-well method (Table 5).

Table 5: Biological screening of extracts from *A. alternata* (UAMH 6385) shake culture.

Extract Description	Extract Mass (mg)	Mushroom Activity
Broth:		
CH_2Cl_2 extract	171	-
EtOAc extract	800	+
EtOH extract	8941	+
Mycelium:		
CH_2Cl_2 extract	2640	-
EtOAc extract	1821	-
EtOH extract	4998	-
*Mycelium after soaking:		
CH_2Cl_2 extract	477	-
EtOH extract	1510	-

*Soaking in acetic acid for 3 days

Isolation of the active metabolite.

Earlier results showed mushroom stimulation in *M. citricolor* from the methanol soluble extract of the culture filtrates of a still culture of *A. alternata* (UAMH 5602) (Table 1). A portion of this extract (4.63 g) was subjected to gel filtration chromatography using Sephadex LH-20 (column 65 g, 36 x 2.8 cm id). The extract was eluted with methanol (20 ml fractions) and eight combinations of successive fractions were made. Each combination was tested for biological activity using the half plate method, and the results are displayed in the following table.

Table 6: Screening of the separated methanol fractions for biological activity.

Test sample	Combined fractions	Total mass (mg)	Bioassay sample concentration (mg/ml)	pH of aqueous test sample	Mushroom stimulation
1	5-9	53	0.417	7.5	-
2	10-11	205	0.412	6.8	-
3	12-16	1092	1.823	5.5	-
4	17-20	1525	1.313	6.1	-
5	20-27	1431	1.692	6.5	+
6	28-34	215	0.417	8.2	-
7	34-42	109	0.417	8.5	-
8	43-50	77	0.200	8.5	-

A portion of test sample 5 (400 mg) was separated using centrifugal counter-current chromatography [39] (solvent system, ethyl acetate-water; mobile phase, ethyl acetate; flow rate, 3 ml/min; spinning rate, 800 rpm). After 1.5 hours, one compound (78 mg) eluted from the column. A yellow colored residue (300 mg) was recovered from the stationary phase after the column was emptied.

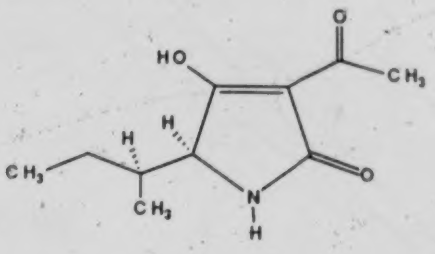
The pure compound and column residue were tested for biological activity using the multi-well method with half strength PDA media. The pure compound (20 mg) and the column residue (53 mg) were each dissolved in 2 ml of water, autoclaved, and 0.200 ml of each solution was pipetted into test wells. Three replicates of each solution were tested. The wells were inoculated with *M. citricolor* (UAMH 6384) and the fungal growth observed during 30 days of diurnal lighting (12 hour photo period) (Table 7).

Table 7: Biological screening of the products from test sample 5 (Table 6).

Description	Concentration (mg/well)	Observations
Purified compound	2.0	- total inhibition of mycelial growth
column residue	5.3	- no activity

The purified compound was identified as tenuazonic acid by comparison of the spectral data to the reported spectra in the literature [28].

Tenuazonic acid:



Tenuazonic acid was obtained as a yellow oil which could not be induced to crystallize: $[\alpha]_D$: -59.0°(c 0.200, CH_3OH); **TLC** R_f: 0.43 (toluene, ethyl acetate formic acid, 6:3:1); **UV** (CH_3OH) λ_{max} (log ε): 240 (3.91), 278 (4.05) nm; **FTIR** (acetone cast): 3200br, 2963m, 2929m, 2924m, 1720m, 1703m, 1698m, 1693s, 1658s, 1650s, 1644s, 1619s, 1614s, 1574s, 1461m, 1453m, 1422m, 1378m, 1224m; **^{1}H NMR** (d-5 pyridine): δ 9.60-9.40 (2H, vbr), 3.95 (1H, d, J = 3.5 Hz), 2.53 (3H, s), 2.07 (1H, m), 1.48 (1H, m), 1.25 (1H, m), 1.03 (3H, J = 7.0 Hz), 0.78 (3H, t, J = 7.5 Hz); **^{13}C NMR** (d-5 pyridine, APT): δ 196.3 (s), 185.8 (s), 176.1 (s), 103.4 (s), 67.1 (d), 37.4 (d), 24.1 (t), 20.8 (q), 16.2 (q), 12.0 (q); **CIMS** (NH_3): m/z 395 (5.7%) $[2M+H]^+$, 198 (100%) $[M+H]^+$; **HREIMS** m/z (relative intensity): 197.1051 found, 197.1052 calcd for $C_{10}H_{15}NO_3$ (0.68), 182 (0.84), 168 (2.4), 149 (8.1), 141 (100), 123 (13.7), 98 (7.4), 86 (10.6), 69 (5.3), 57 (9.3)

Improved procedure for isolation of tenuazonic acid.

When more natural tenuazonic acid was needed for biological studies, it was obtained using the following easier method of isolation [31]. Broth extracts obtained from *A. alternata* were diluted with water and acidified to pH 2 with 5% (v/v) hydrochloric acid.

The solution was extracted exhaustively with dichloromethane and the organic extract was flash chromatographed on silica gel using dichloromethane-acetic acid (9:1 to 6:3). Tenuazonic acid appears to decompose slowly on silica gel since a brown-red band is observed on the column after application of the tenuazonic acid extract, which becomes more appearent as elution procedes. Also, a brown-red outline appears just above the spot observed for tenuazonic acid when silica gel plates are used for thin layer chromatography. Tenuazonic acid is observed as a strong UV absorbing spot on TLC which turns brown when dipped in phosphomolybdic acid developing solution before the TLC plate is charred on a hot plate.

Synthesis of (+/-) tenuazonic acid.

DL- isoleucine (1.31 g, 9.9 mmol) was added to a solution of thionyl chloride (0.82 ml, 9.9 mmol) in methanol (3 ml) at -4 °C. The solution was then refluxed gently for 3 hours, cooled to -4 °C, and triethylamine (1.4 ml, 9.9 mmol) was added to give a thick suspension.* Freshly distilled diketene (0.70 ml, 9.9 mmol) was added dropwise to the stirred suspension and the mixture was stirred for 1 hour. Water was added, the solution was extracted with diethyl ether, and the extract was separated by flash chromatography. Elution with ethyl acetate-Skelly B 1:1 gave the product (1.45 g, 6.3 mmol) which was added to a solution of sodium (0.120 g, 5.4 mmol) in 2 ml of methanol, and the mixture was refluxed for 3 hours. The solution was concentrated under vacuum, water was added and the mixture was extracted with ethyl acetate. The aqueous layer was acidified to pH 2 with 5% HCl and extracted with ethyl acetate again. Evaporation of this ethyl acetate extract yielded (+/-) tenuazonic acid as a pale yellow gum (800 mg, 41%). ^{1}H and ^{13}C NMR analysis revealed the presence of (+/-) allo-tenuazonic acid as approximately 50% of the product mixture.

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* difficult to stir magnetically

(+/-) tenuazonic acid:

$[\alpha]_D$: 0° (*c* 0.276, CH_3OH); **TLC** R_f: 0.43 (toluene, ethyl acetate formic acid, 6:3:1); **FTIR** (acetone cast): 3228br, 2964m, 2934m, 2877 m, 1718s, 1697s, 1659s, 1619s, 1453m, 1422m, 1379m, 1322m, 1291m, 1262m, 1222m. **^{1}H NMR** (d-5 pyridine): δ 9.60 (2H, vbr), 3.96 (1H, d, *J* = 3.0 Hz), 2.53 (3H, s), 2.10 (1H, m), 1.50 (1H, m), 1.26 (1H, m), 1.04 (3H, d, *J* = 7.0 Hz), 0.78 (3H, t, *J* =7.5 Hz); allo-tenuazonic acid: δ 9.60 (vbs), 4.06 (1H, d, *J* = 3.0 Hz), 2.54 (3H, s), 2.11 (1H, m), 1.50 (1H, m), 1.36 (1H, m), 0.85 (3H, t, *J* = 7.0 Hz), 0.83 (3 H, d, *J* = 7.0 Hz); **^{13}C NMR** (d-5 pyridine, APT): δ 196.3 (s), 185.8 (s) ,176.1 (s), 103.4 (s), 67.1 (d), 37.4 (d), 24.1 (t), 20.7 (q), 16.1 (q), 11.9 (q); allo-tenuazonic acid: δ 196.6 (s), 185.9 (s), 176.4 (s), 103.3 (s), 65.6 (d), 37.0 (d), 27.1 (t), 20.6 (q), 16.1 (q), 13.4 (q); **HREIMS** *m/z* (relative intensity): 197.1045 found, 197.1052 calcd for $C_{10}H_{15}NO_3$ (2.4), 182 (1.0), 168 (2.6), 149 (8.6), 142 (7.1), 141 (100), 123 (10), 98 (4.7), 86 (11), 85 (3.2), 57 (3.6).

Synthesis of (+) and (-) tenuazonic acid.

(+) Tenuazonic acid was synthesized from D- isoleucine according to the procedure described for the synthesis of (+/-) tenuazonic acid from DL-isoleucine. The synthetic (+) tenuazonic acid was obtained as an oil (18% overall yield), $[\alpha]_D$ + 32.6° (*c* 1.580, CH_3OH) which was contaminated with allo-tenuazonic acid (^{1}H NMR analysis). Similarly (-) tenuazonic acid was prepared in 9-15% overall yield from L-isoleucine. The synthetic (-) tenuazonic acid was obtained as an oil $[\alpha]_D$ - 9.20° (*c* 0.527, CH_3OH) and was contaminated with allo-tenuazonic acid (^{1}H NMR analysis).

Improved synthesis of (+/-) tenuazonic Acid [29].

Aqueous sodium hydroxide (10% w/v) was added to a suspension of DL-isoleucine (159 mg, 1.21 mmol) in methanol (3 ml) until all the solid dissolved. Freshly distilled diketene (0.50 ml, 7.1 mmol) was added dropwise to the stirred solution and after the addition was complete, the mixture was stirred at room temperature for 1 hour. The solution was then acidified to pH 2 with 10% v/v hydrochloric acid and extracted with dichloromethane. Evaporation of the dried dichloromethane extract gave an oil to which an excess of ethereal diazomethane was added. The solution was allowed to stand at room temperature overnight. Evaporation of the solvent gave an oil which was refluxed in a solution of sodium (75 mg, 3.3 mmol) in methanol (4 ml) for 4 hours. The solution was concentrated, water was added, and the mixture extracted with dichloromethane. The aqueous phase was then acidified to pH 2 with 5% v/v hydrochloric acid and further extracted with dichloromethane. Evaporation of this dried dicloromethane extract gave (+/-) tenuazonic acid (105 mg, 43%) (^{1}H NMR identical to sample prepared previously).

Biological testing of tenuazonic acid.

Solution preparation.

Most of the tenuazonic acid used for biological testing was initially in the non-ionic form except for the first isolation where tenuazonic acid was isolated and tested as a salt (the counter ion of tenuazonic acid was not identified). The non-ionic form of tenuazonic acid is insoluble in water hence, tenuazonic acid was first converted to the ammonium or sodium salt before testing. Solutions of the ammonium salt of tenuazonic acid were prepared for biological testing by delivering the required amount of tenuazonic acid dissolved in dichloromethane into sample vials. The solvent was evaporated, and one drop of concentrated ammonia was added to the vials. The solutions were left for a few hours to allow most of the excess ammonia to evaporate, and then the required amount of water was added to the vials. Solutions of the sodium salt of tenuazonic acid were prepared by adding a solution of a known amount of sodium carbonate to a known amount of tenuazonic acid. When all of the tenuazonic acid had dissolved, the resulting solution was diluted to the desired concentration.

Concentration effects of natural (-) tenuazonic Acid on *Mycena citricolor*.

The multi-well method was used for this bioassay. Aqueous solutions of (-) tenuazonic acid at different concentrations were autoclaved and 0.200 ml portions of each solution were pipetted into test wells. The wells were inoculated with *M. citricolor* (UAMH 6384), and grown under diurnal lighting conditions. Two sets of bioassays were performed, one using full strength and the other using half strength PDA. Observations were made on the bioassays 30 days after inoculation (Table 8).

Table 8: Concentration effects of (-) tenuazonic acid on *Mycena citricolor* after 30 days.

Natural (-) tenuazonic Acid Concentration (mg/well)	Observations on full strength PDA	Observations on half strength PDA
2.00	N/A	- total inhibition of growth
1.00	- total inhibition of growth	- total inhibition of growth
0.50	- strong growth inhibition	+
0.25	+++	+
0.125	+	++
0.063	-	+++
0.032	-	-
0.016	-	-
0.008	-	N/A

+ = mushroom stimulation

Concentration studies of (+/-) tenuazonic acid on *Mycena citricolor* using the multi-well method.

Aqueous solutions of (+/-) tenuazonic acid were autoclaved and 0.150 ml portions of each solution were pipetted into the test wells. The wells were inoculated with *M. citricolor* (UAMH 6384), and grown under diurnal lighting conditions. Two sets of bioassays were performed, one using full strength and the other using half strength PDA. A few days after inoculation, mushroom primordia appeared on some of the inoculation plugs but these did not develop into basidiocarps and one week later they were covered by mycelial growth. Results after three weeks of growth showed inhibition at certain concentrations, but did not show basidiocarp stimulation at any concentration. The cultures were monitored for a further 4 weeks, but nothing new was observed during this time (Table 9).

Table 9: Concentration effects of (+/-) tenuazonic acid on *Mycena citricolor* after 7 weeks using the multi-well method.

(+/-) tenuazonic Acid Concentration (mg/well)	Observations on full strength PDA	Observations on half strength PDA
1.28	strong inhibition	total inhibition
0.638	weak inhibition	strong inhibition
0.319	-	-
0.159	-	-
0.080	-	-
0.040	-	-
0.020	-	-

(-) - indicates absence of mushroom stimulation

Concentration studies of (+/-) tenuazonic acid on *Mycena citricolor* using the half plate method.

Another study was undertaken to obtain a more accurate picture of the active concentration range of (+/-) tenuazonic acid, and to see if better results would be obtained in terms of mushroom stimulation. Divided plates were filled with sterilized full strength PDA media, and media containing (+/-) tenuazonic acid at different concentrations. The plates were inoculated with *M. citricolor* (UAMH # 6384), grown under diurnal lighting conditions (12 hour photo period) and observed for 6 weeks (table 10). Total inhibition was observed above 5.23 x10^{-3}M, but no mushroom stimulation was observed at any concentration.

Table 10: Concentration study of (+/-) tenuazonic acid on *Mycena citricolor* after 6 weeks using the half plate method.

(+/-) tenuazonic acid concentration (x 10^{-3}M)	Observations after 6 weeks of growth
11.5	- Total inhibition of growth
5.23	- Strong inhibition of growth
2.50	- Weak inhibition of growth
1.02	- Weak inhibition of growth
0.808	- Mycelium whiter than control
0.601	- Mycelium whiter than control
0.499	- Mycelium whiter than control
0.392	- Mycelium whiter than control
0.291	- No difference between test and control
0.200	- No difference between test and control
0.100	- No difference
0.094	- No difference
0.079	- No difference

Measured inhibition of mycelial growth of *M. citricolor* by synthetic (+) tenuazonic acid and natural (-) tenuazonic acid.

The effects of the concentrations of synthetic (+/-) and natural (-) tenuazonic acid were examined together under identical environmental conditions. The half plate method was used with half strength PDA media and the plates were inoculated with *M. citricolor* (UAMH 6384). The inhibition of *M. citricolor* by tenuazonic acid was quantified by comparing the rates of growth on the test side with that of the control side. This was done by measuring the diameter of the mycelial growth on each side of the half plates 7 days after inoculation. Three replicates at each concentration were tested, and the average measurements recorded. At this time, mushroom primordia were found on some of the inoculation plugs, but after another week, they were grown over by mycelium. However, mushroom formation was observed on the plates after 25 days of growth . The number of basidiocarps on each of the replicates for each concentration (R_1, R_2, R_3) were counted after 40 days of growth (Table 11). No basidiocarps were observed on any of the controls.

Table 11: Concentration studies of natural (-) and (+/-) tenuazonic acid on the growth and stimulation of *Mycena citricolor*.

Tenuazonic acid concentration (x 10^{-3}M)	Average test diameter (mm)	Average control diameter (mm)	Number of mushrooms after 40 days		
			R_1	R_2	R_3
Natural (-) tenuazonic acid					
4.90	0	58.3	0	0	0
2.41	17.7	54.7	61	46	55
1.21	41.3	56.3	16	11	33
0.62	54.3	57.3	1	0	4
0.39	55.3	57.0	0	0	0
0.16	57.3	57.7	0	0	0
Synthetic (+/-) tenuazonic acid					
4.86	0	62.0	0	0	0
2.22	32.3	60.0	1	0	1
1.32	45.7	57.0	0	0	0
0.67	55.7	58.0	1	1	0
0.44	58.3	58.7	0	15	0
0.22	58.0	59.3	0	2	0

Inhibition studies on synthetic (-) tenuazonic acid.

Synthetic (-) tenuazonic acid was tested for inhibition and for mushroom stimulation of *M. citricolor* in order to verify previous work on the natural compound. The half plate was used in conjunction with half strength PDA media. The plates were inoculated with *M. citricolor* (UAMH 6384) and grown under diurnal lighting conditions. To measure the amount of inhibition, the diameter of the control side was measured against the diameter of the test side of the plate. The measurements were taken 7 and 9 days after inoculation. The results are summarized on the following table. The plates were observed for another 35 days, but no mushrooms were observed during this time.

This experiment may have been affected by the poor quality and control of the lighting in the laboratory. At one point, the light control was turned off and the lights remained on for one entire weekend. Further studies were carried out in a growth chamber where the temperature and the lighting could be controlled more accurately.

Table 12: Inhibition of *M. citricolor* by synthetic (-) tenuazonic acid after 7 and 9 days.

	7 days after inoculation		9 days after inoculation	
(-) tenuazonic acid concentration (x10^{-3}M)	Control diameter (mm)	Test diameter (mm)	Control diameter (mm)	Test diameter (mm)
4.37	56.3	0	71.3	0
2.12	54.0	23.3	70.0	35.3
1.27	55.7	37.0	71.0	51.3
0.815	52.3	47.3	68.7	64.3
0.422	52.3	51.7	69.7	68.3
0.221	53.3	53.0	70.3	69.7

Comparison of stimulation effects of synthetic (-) and (+/-) tenuazonic acid using the multi-well method.

A comparison was made between the stimulating effects of synthetic (-) tenuazonic acid and (+/-) tenuazonic acid, using the multi-well method with half strength PDA media. Solutions of tenuazonic acid in water were autoclaved, and 0.150 ml of each solution was pipetted into one of three replicate wells. The wells were inoculated with *M. citricolor* (UAMH 6384), and placed in a growth chamber. The temperature in the growth chamber was 25°C, and the lighting was set for a 12 hour diurnal photo period. The wells were monitored for 35 days after which the number of mushrooms for each set of concentrations was counted (Table 13).

Table 13: Mushroom stimulation by synthetic (-) and (+/-) tenuazonic acid using the multi-well method

Tenuazonic acid concentration (mg/well)	Number of mushrooms observed
(-) tenuazonic acid	
1.61	0
0.80	17
0.39	7
0.20	5
0.05	3
(+/-) tenuazonic acid	
1.90	0
0.95	1
0.47	15
0.24	6
0.12	6
0.06	4

Study on the effects of synthetic (-), (+) and (+/-) tenuazonic acid.

In many of the previous bioassays, the fungal cultures were grown in a large room where the temperature often fluctuated by 5 or 10°C, and where the lighting conditions were quite often disrupted. In this study, the half plate method was used along with half strength PDA media, and the study was performed in a growth chamber at 25°C under diurnal (12 hour photo period) lighting. Plates containing (+), (-) and (+/-) tenuazonic acid solutions were prepared at different concentrations and inoculated with *M. citricolor* (UAMH 6384). The plates were measured for inhibition 8 days after inoculation by comparing the diameter of mycelial growth of the control side with the diameter of mycelial growth of the test side. The number of fruiting bodies observed for each solution was counted 29 days after inoculation. These results are tabulated in the following table.

Table 14: Effects of synthetic (-), (+) and (+/-) tenuazonic acid on *Mycena citricolor*

Tenuazonic acid concentration (mg/ml)	(x 10^{-3}M)	Average control diameter (mm)	Average test diameter (mm)	Average number of mushrooms
(+) tenuazonic acid				
0.671	3.40	45.7	16.3	10.3
0.501	2.54	49.3	33.0	2.7
0.336	1.71	51.6	48.3	2.7
0.168	0.85	64.0	62.7	4.3
0.072	0.36	63.0	62.7	1.3
(+/-) tenuazonic acid				
0.667	3.39	65.3	0.0	0.0
0.499	2.53	63.7	14.3	25.3
0.333	1.69	61.0	40.7	13.3
0.166	0.84	51.6	47.0	28.3
0.068	0.35	64.3	63.7	0.0
(-) tenuazonic acid				
0.667	3.39	56.7	0.0	0.0
0.502	2.55	49.0	0.0	0.0
0.333	1.69	51.7	13.0	19.0
0.168	0.85	45.7	28.3	29.0
0.069	0.35	62.0	57.3	40.0

Mushroom stimulation of other strains of *Mycena citricolor*.

Most of the previous bioassays were performed using *M. citricolor* (UAMH 6384) isolated from *Erogoga acuminata* , a medicinal plant. Since we were originally interested in the strains of *M. citricolor* isolated from coffee plants, four coffee isolates of *M. citricolor* were tested for mushroom stimulation by synthetic (-) tenuazonic acid. The multi-well method was used in conjunction with half strength PDA media. The plates were grown under diurnal lighting conditions (12 hour photo period) at 25°C. The number of mushrooms observed 20 days after inoculation is tabulated below for the 4 different strains of *Mycena citricolor* tested.

Table 15: Mushroom stimulation of other strains of *Mycena citricolor*.

(-) Tenuazonic acid concentration (mg/well)	Total number of mushrooms observed after 15 days of growth			
	MCH2	MCH4	MCH9a	MCH12
1.85	+0	^0	+0	^0
0.99	4	*0	0	+0
0.57	15	*0	0	18

* mushroom primordia observed
^ total inhibition of mycelial growth
+ strong inhibition of mycelial growth

Mycena citricolor versus other fungi.

The strains of *Mycena citricolor* available in this lab were tested against *Talaromyces flavus* (UAMH 4890) and *Penicillium oxalicum* (UAMH 5148), two fungi reported to induce stimulation. Each of the strains of *M. citricolor* and one of the other fungi were co-inoculated on opposite sides of culture plates containing half strength PDA media. The plates were grown in diurnal lighting (12 hour photo period) at 25 °C for 40 days and monitored for mushroom stimulation and mycelial inhibition (Table 16).

Table 16: *Mycena citricolor* versus *Penicillium oxalicum* and *Talaromyces flavus*.

Mycena citricolor	*Penicillium oxalicum*		*Talaromyces flavus*	
strain	Mushroom stimulation	Mycelial inhibition zone	Mushroom stimulation	Mycelial inhibition zone
UAMH # 6384	+	+	+	-
MCH2	-	+	±	-
MCH4	-	+	-	-
MCH9a	-	+	-	-
MCH12	-	+	-	-

Analysis of some broth extracts of *P.oxalicum* and *T. flavus*.

Shake and still cultures of *T. flavis* and *P. oxalicum* each were grown in 5 litres of full strength Potato Dextrose Broth (PDB). After one week of growth, the cultures were sterilized by autoclaving, and then the mycelium was filtered from the broth. The broths were concentrated to 1/10 volume, and a portion (60 ml) of each broth concentrate was basified to pH 9 with aqueous ammonia. The basic solutions were extracted with dichloromethane, the organic layer discarded and the aqueous phase acidified to pH 2 with 5% v/v hydrochloric acid and extracted with ethyl acetate.

The ethyl acetate extracts were tested for the presence of tenuazonic acid by TLC analysis. Tenuazonic acid forms a dark brown spot on silica gel TLC plates when dipped in phosphomolybdic acid solution before charring on a hot plate. However, tenuazonic acid was not detected in any of these acidic extracts of any of these cultures.

The broths and the acidic fractions from these different cultures were tested for mushroom stimulation and for mycelial inhibition of *Mycena citricolor*. The multi-well method was used for analysis using full strength PDA. The plates were inoculated with *M. citricolor* (UAMH 6384) and grown in under diurnal lighting (12 hour photo period) for 30 days at 25°C. Table 17 displays these results.

Table 17: *Mycena citricolor* versus culture fluid extracts of *Penicillium oxalicum* and *Talaromyces flavus*.

Broth description			Mushroom stimulation	Mycelial inhibition
P. oxalicum				
	Shake culture	concentrated broth	-	+
		acidic fraction	-	-
	Still culture	concentrated broth	mushroom primordia	+
		acidic fraction	mushroom primordia	-
T. flavus				
	Shake culture	concentrated broth	-	+
		acidic fraction	-	-
	Still culture	concentrated broth	-	+
		acidic fraction	-	-

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